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THE UNIVERSITY OF ALBERTA

REGULATION OF DEOXYRIBONUCLEOTIDE METABOLISM

by

Ⓒ DAREL HUNTING

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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To Bonnie

ABSTRACT

The fundamental aim of this research was to study the relationships between changes in ribonucleoside triphosphate and deoxyribonucleoside triphosphate concentrations and biological parameters such as growth inhibition and cell death.

Several methods for measuring deoxyribonucleoside triphosphate concentrations were reviewed and it was concluded that the enzymatic assay was best suited to this project. However, in order to obtain a sufficiently sensitive and accurate assay, it was necessary to make extensive modifications to published procedures.

A quantitative analysis of the metabolism of radioactive purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides in CHO cells was made and deoxyribonucleoside mono-, di- and triphosphate pools were measured.

Incorporation of radioactivity into DNA and RNA from radioactive thymidine and uridine, respectively, was shown not to be a reliable measure of the rates of synthesis of these macromolecules. Consequently, a better method was developed and tested. This involved correcting the rates of radioactive precursor incorporation into nucleic acids for the specific activity of the immediate precursor pool. Then, this method was used to measure the net rate of DNA and RNA synthesis in Chinese hamster ovary cells treated with pyrazofurin or mycophenolic acid. Finally, this method was used to determine the rate of flux through the ribonucleotide reductase reaction.

Several models for allosteric regulation of ribonucleotide reductase in whole cells were reviewed in preparation for studying the regulation of ribonucleotide reduction in intact cells.

A study of the control of ribonucleotide reduction by deoxyribonucleoside triphosphates in intact CHO cells demonstrated that none of the published ribonucleotide reductase models adequately explain deoxyribonucleoside triphosphate pool size changes in whole cells. Further study revealed that ribonucleotides played an important role in determining deoxyribonucleotide concentrations, but the actual mechanism involved was not determined.

Finally, the effect of nucleotide pool size changes on growth rate, cell viability and progression through the cell cycle was determined. Although some clues were obtained, the mechanism of cell death induced by nucleotide pool size changes could not be determined.

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LIST OF ABBREVIATIONS

A	adenine
ADP	adenosine diphosphate
AdR	deoxyadenosine
AMP	adenosine monophosphate
AR	adenosine
ATP	adenosine triphosphate
C	cytosine
CDP	cytidine diphosphate
CdR	deoxycytidine
CMP	cytidine monophosphate
CR	cytidine
CTP	cytidine triphosphate
dADP	deoxyadenosine diphosphate
dAMP	deoxyadenosine monophosphate
dATP	deoxyadenosine triphosphate
dCDP	deoxycytidine diphosphate
dCMP	deoxycytidine monophosphate
dCTP	deoxycytidine triphosphate
dGDP	deoxyguanosine diphosphate
dGMP	deoxyguanosine monophosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
DNA-A	DNA-adenine
DNA-C	DNA-cytosine
DNA-G	DNA-guanine

List of Abbreviations (Continued)

DNA-T	DNA-thymine
dTDP	deoxythymidine diphosphate
dTMP	deoxythymidine monophosphate
dTTP	deoxythymidine triphosphate
dUDP	deoxyuridine diphosphate
dUMP	deoxyuridine monophosphate
dUTP	deoxyuridine triphosphate
H	hypoxanthine
G	guanine
GDP	guanosine diphosphate
GdR	deoxyguanosine
GMP	guanosine monophosphate
GR	guanosine
GTP	guanosine triphosphate
HPLC	high performance liquid chromatography
IMP	inosine monophosphate
NAD	nicotinamide adenine dinucleotide
O.D.	optical density
PALA	N-(phosphonoacetyl)-L-aspartate
PCA	perchloric acid
$^{32}P_i$	^{32}[orthophosphate]
poly[d(AT)]	poly-deoxy (adenylate: thymidylate)
poly[d(IC)]	poly-deoxy (inosinate: cytidylate)
RNA	ribonucleic acid
RNA-A	RNA-adenine

List of Abbreviations (Continued)

RNA-C	RNA-cytosine
RNA-G	RNA-guanine
RNA-U	RNA-uracil
T	thymine
TCA	trichloroacetic acid
TdR	thymidine (deoxythymidine)
U	uracil
UDP	uridine diphosphate
UdR	deoxyuridine
UMP	uridine monophosphate
UR	uridine
UTP	uridine triphosphate

CHAPTER 1

GENERAL INTRODUCTION

Our understanding of growth inhibition and cell death resulting from drug-induced changes in the concentrations of purine or pyrimidine ribo- and deoxyribonucleotides has steadily increased as a result of efforts to develop more effective cancer chemotherapeutic agents. However, in many cases, it is still not known how changes in nucleotide concentrations inhibit cell growth, and very little is known about the sequence of events which leads to loss of cell viability under these conditions.

Agents which specifically affect nucleotide metabolism may be divided into two groups: those which exert their primary effect on ribonucleotide metabolism such as mycophenolic acid and adenosine, and those which exert their primary effect on deoxyribonucleotide metabolism, such as thymidine.

Growth inhibition and cell death induced by agents affecting deoxyribonucleotide metabolism are generally explained in terms of inhibition of ribonucleotide reductase which results in a decreased supply of one or more deoxyribonucleoside triphosphates; inhibition of DNA synthesis and ultimately cell death follow. With agents that affect ribonucleotide metabolism directly, it is usually postulated that changes in the concentrations of ribonucleotides result in a decreased supply of one or more deoxyribonucleotides leading to an inhibition of DNA synthesis and cell death.

The aim of this research was to study the proposed links among ribonucleoside triphosphate and deoxyribonucleoside triphosphate pool size changes, growth inhibition and cell death.

Because deoxyribonucleoside triphosphates are present in picomolar amounts per 10^6 cells, very sensitive assays are required for their routine measurement, especially in drug treated cells. Therefore, from a review of the methods used for the measurement of deoxyribonucleoside triphosphates in cells (Chapter 2), it was concluded that an enzymatic assay for deoxyribonucleotide triphosphate was best suited for this project. In order to obtain a sufficiently sensitive and accurate assay, it was necessary to choose the best features from the more than 20 different assays already published, and as well to introduce new features (Chapter 3).

In preparation for manipulating nucleotide pools using naturally occurring bases and nucleosides, and more importantly in preparation for accurately measuring the rate of flow of metabolites via ribonucleotide reduction and RNA and DNA synthesis, the metabolism of radioactive purine and pyrimidine bases, ribo- and deoxyribonucleosides was studied in Chinese hamster ovary cells (Chapters 4 and 5).

Considerable evidence in the literature indicated that the commonly used simple radioisotopic incorporation methods for measuring rates of DNA and RNA synthesis were unreliable; therefore, a more reliable method involving the correction of the radioisotope incorporation for the specific activity of the precursor pool was used to measure these rates in control and drug treated cells. This method was also used as the basis for determining the flux through the ribonucleotide reductase reaction (Chapter 6).

Ribonucleotide reductase is commonly believed to be a key enzyme in the control of deoxyribonucleotide synthesis and possibly in the control of DNA synthesis (1). There is enzymological evidence that this enzyme is allosterically controlled and more than ten models have been proposed to explain the regulation of ribonucleotide reduction and hence the regulation of deoxyribonucleotide concentrations in whole cells. As well, these models are often used to explain the changes in deoxyribonucleotide pool sizes caused by drugs that affect nucleotide metabolism. The models are all quite different and there is no a priori reason to choose one over another; in some cases, none of the models can explain deoxyribonucleotide pool sizes in whole cells (2). The published models for the regulation of ribonucleotide reductase in whole cells are reviewed and the experimental evidence for and against each model is considered in Chapter 7.

A study of the effects of changes in ribonucleotide concentrations on deoxyribonucleotide concentrations is reported in Chapter 8. The next step was to determine if any of these models is sufficient to explain experimentally observed interrelationships among concentrations of the several deoxyribonucleoside triphosphates in whole cells (Chapter 9). Finally, the relationship between nucleotide pool sizes and growth rate, cell viability, and progression through the cell cycle was studied (Chapter 10).

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CHAPTER 2

METHODS FOR THE DETERMINATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATE CONCENTRATIONS

INTRODUCTION

Deoxyribonucleoside triphosphates are substrates for DNA synthesis, and hence are essential for cell multiplication as well as for DNA repair. Thus their concentrations in cells are of interest in relation to the regulation of DNA synthesis and repair and to the action of a number of cancer chemotherapeutic agents that affect nucleotide metabolism. In addition, altered concentrations of deoxyribonucleoside triphosphates have been implicated in certain human immunodeficiency diseases and in the toxicity of certain deoxyribonucleosides.

The deoxyribonucleoside triphosphates are present in cells at very low concentrations, and are not readily separated from the corresponding ribonucleotides; hence they were not detected in early studies of nucleotide metabolism and nucleotide concentrations in cells. Their existence had been demonstrated by the mid-1950's however, and thereafter a number of approaches to their assay in cell extracts was undertaken. In all, six general methods have been developed: microbiological assay, isolation by conventional chromatography and detection spectrophotometrically, isotope-dilution in intact cells, labeling with radioactive orthophosphate, high performance liquid chromatography, and enzymatic assay, using DNA polymerase. Several appreciably different variants of each general method exist.

This review describes the principal methods for the measurement of deoxyribonucleoside triphosphates, and evaluates them critically. Its purpose is a) to provide an assessment of the factors that are important in each method, so that published deoxyribonucleoside triphosphate measurements can be critically evaluated; b) to help investigators chose the method best suited to their need, and c) to indicate where further improvements may be necessary or desirable.

For each of the six major types of deoxyribonucleoside triphosphate assays the general principle and historical development of the method will be presented, and its advantages and disadvantages considered. As an introduction, however, problems encountered in extracting these nucleotides from cells will be discussed.

EXTRACTION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

Deoxyribonucleoside triphosphate values in the literature are probably influenced as much by the method of extraction as by the method of assay. Cell extraction methods in general have been reviewed by Hauschka (review: 1) and will not be discussed here in detail. However some points that are relevant to the problem at hand include:

1) The washing of cells prior to extraction is not only unnecessary but unwise. As pointed out by Hauschka, washing can result in the hydrolysis of nucleoside tri-, di-, and monophosphates (review: 1). Tyrsted thoroughly studied the effects of washing cells on the deoxyribonucleotide triphosphate content of cells and found that a single wash with ice-cold medium or isotonic sodium chloride resulted in a 43-80% loss of deoxyribonucleoside triphosphates (2). This was confirmed by Kinahan et al. (3), and by Walters et al., who also found that washing the cells caused deoxyribonucleotide breakdown though exact values were not given (4).

2) PCA extraction usually gives the highest yield of nucleotides (review: 1).

3) 0.4 M is the optimal PCA concentration for extraction (5).

4) 5-10% TCA will extract cellular nucleotides, but the yields are not as reproducible as with PCA, possibly

because TCA does not efficiently extract nucleotides bound to intracellular polycations (review: 1).

5) 60% methanol or 70% ethanol have been used to extract nucleotides from cells with the advantage of being readily lyophilized (review: 1).

North _et al_. recently have reported that several enzymes that interfere with the enzymatic assay of deoxynucleoside triphosphates are present in 60% methanol extracts of cultured HeLa cells (6). These activities included a nuclease, nucleoside diphosphate kinase, and deoxynucleoside monophosphate kinases which can phosphorylate dAMP, dGMP, and dCMP. They suggest that methanol extraction of cells will give artificially high values for the deoxyribonucleoside triphosphates when assayed enzymatically because of degradation of the DNA template and phosphorylation of the degradation products to deoxyribonucleoside triphosphates by the contaminating enzymes. Although their findings will not be reviewed here in detail, there were some inconsistencies in the results. For example, they did not observe any exonuclease activity during the assay of standards, but found this activity during the assay of the methanol extracts, although the _E. coli_ DNA polymerase I, which was used in their assay, contains both 5'→3' and 3'→5' exonuclease activities as part of the same enzyme. Furthermore, the small amount of deoxynucleoside monophosphate kinase activity that they observed is inadequate to explain the 500% increase in the apparent amount of deoxyribonucleoside triphosphates in methanol extracts as compared to PCA extracts.

Nevertheless, methanol extraction of cells will have to be carefully re-examined to determine if it introduces errors into the enzymatic assay. In fact, contaminating enzymes such as phosphatases can result in errors, regardless of the assay method used.

6) If cell extracts are lyophilized and redissolved in a known volume, then the recovery of the nucleotides through the overall process should be measured. If cells are extracted with a known volume of extraction medium and a portion of this extract is used for analysis, then dilution of the extract by cellular water and by medium around the cells should be determined.

7) Even if the best extraction method and the greatest care are used, it is still important to assess the quality of the final extract. While this is not an exact parameter, we have found that the nucleoside triphosphate to diphosphate ratio is a sensitive indicator of nucleotide breakdown in the cells (D. Hunting and J.F. Henderson, unpublished). We therefore routinely measure the ATP/ADP ratio, as determined by HPLC, and consider a value of 10 or more for cultured cells to be an indication of satisfactory extraction and sample handling technique. Excessive manipulation of cells, such as washing prior to extraction, an inappropriate extraction medium, failure to keep the extract cold, or to neutralize it within one hour may significantly

lower the ATP/ADP ratio.

A few examples may be given to indicate how ATP/ADP ratios may vary. In two extracts made using cold 0.4 M PCA these ratios were 11.0 and 13.5, and in another extract using 60% methanol at 30°C for 10 min followed by storage overnight at -20°C, it was 12.6. However, when the extraction was made using 60% methanol at -20°C overnight, the ratios for two samples were 5.8 and 3.9.

To show the different extraction procedures that have been used, Table 1 compares various procedures used to prepare cell extracts for enzymatic assays. The majority of methods do not involve washing cells, but there are a few exceptions, one of which included three washings. Since Tyrsted reported a value of 60% for the average amount of deoxyribonucleoside triphosphate breakdown from a single wash, three washes could result in a recovery of only 6% of the original deoxyribonucleoside triphosphates present in the cell. The degree of breakdown during cell washing probably varies from cell type to cell type, but one should regard nucleotide values obtained from washed cells with some caution.

Most of the extraction media were used at optimum concentrations. The majority of procedures did not include the measurement either of recovery or of dilution. Rarely were attempts made to minimize dilutions by wiping media from the inside of the centrifuge tube before extraction. Finally, the quality of the extracts was rarely

Table 1

Methods used to extract deoxyribonucleoside triphosphates

Cells washed before extraction	Extraction medium	Dilution or recovery measured	Quality of extract assessed	Reference
No	60% MeOH	Recovery	No	7
No	PCA	No	No	8
No	60% MeOH	Recovery	No	9
No	1.0 M PCA	No	Yes	3
No	60% EtOH	Recovery	Yes	2
No	60% MeOH	Recovery	Yes	10
1X	66% EtOH	Recovery	Yes	11
2X	0.5 M PCA	No	No	12
No	0.5 M PCA	No	No	13
1X	60% MeOH	No	No	14
No	0.5 M PCA	No	No	15
No	2.0 M PCA	No	No	16
No	0.5 M PCA	No	No	17
No	0.4 M PCA	Dilution	Yes	18

(... cont'd.. Table 1 ...)

Cells washed before extraction	Extraction medium	Dilution or recovery measured	Quality of extract assessed	Reference
1X	0.5 M PCA	No	No	19
No	2.0 M PCA	No	No	20
2X	0.5 M PCA	No	No	21
1X	60% MeOH	No	No	22
3X	5% TCA	No	No	23
No	0.5 M PCA	No	No	24
No	5% TCA	No	No	25
No	0.4 M PCA	No	No	26
No	60% MeOH	No	No	27
No	0.4 M PCA	Dilution	Yes	a

a) D. Hunting and J.F. Henderson, unpublished; see Appendix.

assessed.

MICROBIOLOGICAL ASSAY

The microbiological assay for deoxyribonucleotides was introduced in 1957 and subsequent modifications resulted in an accurate assay of high specificity, but of relatively low sensitivity. Although the last reported use of the microbiological assay was more than 10 years ago, it will be considered in this review because it still is a valid technique, which played an important role in the early studies of cellular deoxyribonucleotide pools, and because some of the results obtained with this method have not been repeated using other assay methods and are still being cited (review: 28). Therefore this assay will be discussed in the context of all of the presently used assay methods in terms of its accuracy, sensitivity, reproducibility and problems or possible pitfalls.

General Principle:

The microbiological assay is based on the principle that certain organisms, such as *Lactobacillus acidophilus*, require exogenous deoxyribonucleosides or deoxyribonucleoside monophosphates for growth. Standard curves can be constructed by plotting cell number, or more commonly, turbidity at approximately 650 nm, after 24-36 hr of incubation at 37°C, against the amount of deoxyribonucleoside standard in each culture. The standard curves are linear up to approximately 2 nmoles/tube but then flatten off as maximum growth stimulation is approached. Deoxyribonucleoside di- and triphosphate concentrations can be determined by converting these compounds to the deoxyribonucleosides with phosphatases. Some specificity can be obtained by hydrolyzing the purine deoxyribonucleosides under mild acidic conditions; thus, values for the total purine and the total pyrimidine deoxyribonucleoside content can be obtained. Further specificity can be achieved by chromatographing the cell extracts to separate the individual deoxyribonucleosides, and then assaying each one separately.

Development of the Assay:

The origins of the microbiological determination of deoxyribonucleotides lie in an assay for DNA which was developed by Hoff-Jorgensen in 1952 (29). This assay was based on the fact that DNA would support the growth of the deoxyribonucleoside requiring bacterium *Lactobacillus acidophilus* R26, after hydrolysis of the DNA to monophosphates with DNase. Five years later, Hoff-Jorgensen published an assay for deoxyribonucleoside monophosphates, based on his 1952 method for DNA, and he also demonstrated that hydrolysis of deoxyribonucleoside monophosphates to deoxyribonucleosides with phosphatase did not change the results (30). The phosphatase was not used routinely, nor did he determine the ability of the assay to measure deoxyribonucleoside di- and triphosphate concentrations. Separate values for purine and pyrimidine deoxyribonucleoside monophosphates were determined by first measuring the total deoxyribonucleoside monophosphate content, and then hydrolyzing the purine derivatives under mild acidic conditions and assaying again for the pyrimidine deoxyribonucleoside monophosphate content.

In contrast to Hoff-Jorgensen's results, Siedler *et al*. found that the sensitivity of this assay varied with different deoxyribonucleoside monophosphates, and that the sensitivity to monophosphates was much less than to deoxyribonucleosides. They suggested that the assay would be improved by treating all samples with phosphatase (31). Schneider and Potter studied the method further and reported that *Lactobacillus acidophilus* R26 could grow on medium supplemented with deoxyribonucleosides or deoxyribonucleoside monophosphates, but not on diphosphates or triphosphates (32). They also found that the procedure of Hoff-Jorgensen, which required autoclaving the medium and extracts prior to the addition of the bacterial innoculum, resulted in the hydrolysis of nucleoside di- and triphosphates, so that the bacteria could grow on media supplemented with these compounds; previous measurement of the deoxyribonucleoside and deoxyribonucleoside monophosphate content of tissue extracts (33) probably were too high because of deoxynucleoside di- and triphosphate breakdown during autoclaving. Schneider and Potter recommended that filtration should be substituted for autoclaving and that the deoxynucleoside di- and triphosphate content of tissue extracts could be determined by enzymatic hydrolysis of these compounds before preforming the assay (33). In 1959 Siedler and Schweigert clarified the question whether or not deoxyribonucleoside monophosphates were used as readily as deoxyribonucleosides by *Lactobacillus acidophilus* when they reported that ribonucleotides, if present in the assay medium, inhibited the utilization of deoxyribonucleoside monophosphates but not of deoxyribonucleosides (34). This explained why different laboratories had reached different conclusions regarding the utilization of deoxyribonucleoside monophosphates. In 1962, Schneider confirmed these results and decided that the best routine assay procedure was to hydrolyze all the ribo- and deoxyribonucleoside phosphates with phosphatase to remove interference by ribonucleotides and allow the assay of all the deoxyribonucleoside derivatives in extracts (35).

In 1963, Larsson demonstrated that either *L. acidophilus* R-26 or *L. leichmannii* could be used in the assay, although when *L. leichmannii* was used, it was necessary to remove all vitamin B_{12} from the medium and extracts since this organism can be grown on medium supplemented with either vitamin B_{12} or deoxyribonucleosides (36).

Finally, in 1966, Brown and Handschumacher used the fully developed assay to measure deoxyribonucleotide pools in *Streptococcus fecalis*. Their procedure also included a further refinement in that they chromatographically separated all the deoxyribonucleoside triphosphates, then hydrolyzed each with phosphatase and assayed them separately (37).

In conclusion, this method is specific for deoxyribose compounds and the specificity can be increased by chromatographically separating the compounds before assaying them. This method allows the measurement of amounts of deoxyribonucleotides greater than 0.5 nmoles, with a standard error of approximately 5% (32). The main problems with this technique are that it is quite insensitive compared to more recently developed techniques (e.g., the enzymatic assay is as much as 1000 times more sensitive); it is also very laborious, especially if the amount of each deoxyribonucleoside triphosphate, rather than their sum total, is to be determined.

CHROMATOGRAPHIC - SPECTROPHOTOMETRIC ASSAY

The first reported use of chromatographic isolation followed by uv measurement to quantitatively determine deoxynucleoside triphosphate concentrations in cell extracts was in 1955 (38,39), the same year the microbiological assay was introduced. Although the chromatographic method has not been used frequently, it has provided valuable information on topics such as adenosine toxicity and thymine-less death (40,41,42). Its most recent use was in 1977 in a study of thymidine triphosphate concentrations in malignant tumors (42). The main drawbacks of this method are that it is laborious and relatively insensitive.

General Principle:

The principles upon which the chromatographic assay is based are as follows:

1) Separation: Deoxyribonucleotides can be isolated from cell extracts using anion exchange column chromatography. However, complete purification of the deoxyribonucleoside triphosphates requires further chromatography on paper. Although one-step purification of the deoxyribonucleotides from cell extracts is possible using two-dimensional thin-layer chromatography, its usefulness is limited because not enough deoxyribonucleotide can be isolated to permit accurate measurement; however there is one published procedure in which this method has proved useful (42,43).

2) Quantitative Measurement: Three methods which have been used to determine the amount of each deoxyribonucleotide after chromatographic separation are: measurement of uv absorbance, the luciferase assay, and bioassay, with the measurement of uv absorbance being the most common method used. The bioassay has been discussed separately and will only be mentioned briefly here. The luciferase assay has only been used in one study (40), and all four deoxyribonucleoside triphosphates could be assayed by this method.

Development of the Assay:

Potter reported the quantitative measurement of pyrimidine deoxynucleotides in calf thymus extracts in 1955 (38,39) and gave a complete description of the method in 1957 (44). The basic procedure was as follows: neutralized, concentrated tissue extracts were chromatographed on an anion exchange column and the nucleotides were eluted using a gradient. Fractions were collected and the uv absorbance was determined. Preliminary identification of the fractions was based on the A_{275} to A_{260} ratio. The deoxyribonucleotides did not separate well from the ribonucleotides, and a second column chromatographic procedure followed by paper chromatography was necessary to isolate pure dTTP and dCTP. The amounts isolated were determined by uv absorbance, with identification of the compounds based on deoxyribose and phosphorous content, and on spectral data. No purine deoxyribonucleotides could be isolated by this technique, and data on accuracy and sensitivity were not reported.

In 1957 LePage used a similar procedure to determine the dATP concentration of rat tumor extracts (45). ATP and dATP were separated from the other nucleotides by anion exchange chromatography and after desalting on a charcoal-Celite column, dATP was separated from ATP by 3 sequential runs on paper chromatograms. The amount of pure dATP isolated was 2 μmoles, and the recovery determined by using a dATP standard, was 50 to 67%.

In 1962, Klenow used a substantially different procedure to measure dATP concentrations in extracts of Ehrlich ascites tumor cells (40). He simply chromatographed the cell extracts on Whatman No. 40 filter paper, eluted the dATP and determined its absorbance. Its identity was confirmed from spectral data and by using the diphenylamine reaction. One disadvantage of this method was that the chromatography step required 75 h which could have resulted in dATP hydrolysis. He did not report values for dATP recovery.

Klenow also introduced an ingenious modification to the assay by using luciferase as well as uv measurements to measure the amount of deoxyribonucleoside triphosphate that had been purified by paper chromatography (40). Values for the sensitivity and accuracy of the determinations were not given. Although the luciferase assay is a convenient method of determining deoxyribonucleoside triphosphates after chromatography, no other reports of its use for this purpose could be found. The luciferase assay was originally used to determine dATP in extracts that had been treated with periodate to oxidize ATP (40,46). However, since all the deoxyribonucleoside triphosphates can be measured by the luciferase assay, it is probable that this technique measured the total deoxyribonucleoside triphosphate concentration and not just that of dATP.

In 1963, Potter and Nygaard used the chromatography-uv measurement procedure of Potter _et al_. (44) to measure dTTP concentrations in extracts of rat spleen and thymus (47). However, they found the uv measurements were too insensitive to measure dTTP in thymus extract so they used the microbiological assay instead. Although they did not report values for sensitivity, the lowest amount of dTTP determined was 19 nmoles.

The assay was improved in 1969 by Bücher and Oakman who introduced both two dimensional thin-layer chromatography and isotope-dilution (43). The thin-layer chromatographic method, based on the procedure of Randerath and Randerath (48), involved one-dimensional thin-layer chromatography on PEI-cellulose plates. Although a single sample was streaked across the origin of three plates, the plates were heavily overloaded and the dTTP that was isolated had to be purified by rechromatographing on PEI cellulose, using a two-dimensional system. The dTTP was then eluted and measured spectrophotometrically.

The isotope-dilution technique involved the addition of [^{14}C]dTTP of a known specific activity to the PCA before extracting the tissue. After isolating the dTTP as described above, the endogenous dTTP content was calculated from the reduction of the specific activity of [^{14}C]dTTP. This assay method was simpler than previous chromatographic-spectrophotometric procedures, and the use of the isotope-dilution technique automatically corrected for loss of dTTP by incomplete recovery or breakdown during the isolation procedure. In principle, this method should have been quite accurate; however, no values for accuracy were reported.

The most recent use of the chromatographic-spectrophotometric assay was in 1977 (42). The method utilized two-dimensional thin-layer chromatography, as described above, followed by determination of dTTP by uv absorbance. A minimum sensitivity can be determined from the fact that 400 pmoles of dTTP was detected in an extract of 10^7 cells. Thus if all the extract was chromatographed, the sensitivity of the assay would be less than 400 pmoles.

In conclusion, although the chromatographic-spectrophotometric assay is quite laborious, it has the advantages of being direct and accurate, especially if the recovery is measured or if the isotope-dilution technique is used.

ISOTOPE DILUTION IN INTACT CELLS

The method of measuring intracellular nucleotide pool sizes by applying the isotope-dilution principle to intact cells was developed by Forsdyke (49). Deoxyribonucleoside triphosphate pool size measurements using this method have only been made in two laboratories, that of Forsdyke himself (49-53) and that of R.L.P. Adams (54). It must be clearly understood that although the isotope-dilution principle is valid in itself, the assumptions involved in applying it to the measurement of intracellular nucleotide pools have not been properly tested. Few changes have been made in Forsdyke's original procedure, although it has been applied to different types of problems. For this reason, the development of the method will not be discussed separately.

General Principles:

The isotope dilution assay of Forsdyke is based on the isotope dilution principle and probably also on the enzymatic isotope-dilution method developed by Newsholme and Taylor (55), Brooker and Appleman (56), and Grander (57). The assumptions and reasonings of this method will first be stated as clearly as possible, and then critically evaluated.

1) If cells are incubated *in vitro* with a radioactive deoxyribonucleoside precursor of DNA, then the rate of incorporation of radioactivity into DNA will be proportional to the specific activity of the precursor, and to the V_{max} of the rate-limiting step in the pathway.

2) It is assumed that the specific activity of the deoxyribonucleoside precursor will be reduced by any compounds (nucleosides and nucleotides), either extracellular or intracellular, which enter the pathway of metabolism and incorporation of the precursor prior to the rate-limiting step; therefore the total pool of these compounds will be measured through isotope dilution. The total amount of these compounds normally present in the cells and medium is called the "intrinsic pool", while the total amount of these compounds which might be added to the medium experimentally is called the "extrinsic pool".

3) It is also assumed that compounds which enter the pathway after the rate-limiting step will not reduce the specific-activity of the external deoxyribonucleoside precursor and therefore will not be detected by the isotope-dilution assay.

4) Operationally, if a constant quantity of radioactive precursor is added to the medium, along with varying quantities of the same, non-radioactive precursor, then a plot of the reciprocal of the radioactivity incorporated into DNA (abscissa) against the total concentration of added precursor (extrinsic pool) will be linear. The slope of this plot is taken to be a measure of the V_{max} of the rate-limiting step, while the negative intercept at the ordinate is taken to be a measure of the intrinsic pool (which, as stated above, may include both intracellular and extracellular compounds).

5) A change in the position of the rate-limiting step in the pathway, as induced for example, by treatment with drugs, will change the number of compounds which reduce the specific activity of the radioactive deoxyribonucleoside precursor, and therefore will change the size of the intrinsic pool.

Analysis of the Method:

Two especially important assumptions involved in the use of the isotope-dilution method for the measurement of intracellular nucleotide pools, remain untested:

1) It is assumed that both intracellular deoxyribonucleotide pools and extracellular non-radioactive deoxyribonucleoside precursors reduce the incorporation of added radioactive deoxyribonucleoside precursor into DNA by exactly the same mechanism; i.e. it is assumed that intracellular deoxyribonucleotides can reduce the specific activity of the extracellular deoxyribonucleoside via a rapid chemical equilibrium. Therefore, if a radioactive deoxyribonucleoside were incubated with cells and then isolated from the medium, its specific activity should have decreased by an amount dependent on the size of the intrinsic pool, which, depending on the position of the rate-limiting step in the pathway, could include intracellular nucleotides as well as nucleosides. There is no evidence that this assumption is valid. Furthermore, it is likely that this point has caused some confusion, as generally when one refers to the dilution of, for example, radioactive thymdidine by

intracellular thymidine nucleotides it is implied that the dilution occurs only within the cells, and only after the radioactive thymidine has been converted to nucleotides.

2) It is assumed that intracellular nucleotide pools and synthetic pathways which enter the pathway under study distal to the rate-limiting step, have no effect on the incorporation of the extracellular radioactive precursor into DNA. Thus for example, in a situation in which the phosphorylation of thymidine is the rate-limiting step for the incorporation of radioactive thymidine into DNA, it is assumed that the rate of incorporation of radioactivity into DNA is independent both of the size of the thymidine nucleotide pool and of the rate of thymidylate synthesis de novo. This is contrary to what one would intuitively predict, and it is therefore difficult to accept this assumption without any supporting experimental evidence.

Sjöstrom and Forsdyke have reported that at thymidine concentrations less than 5 μM, the rate-limiting step for the incorporation of radioactive thymidine into DNA in cultured thymus cells was thymidine kinase (51). Therefore, according to the isotope-dilution theory of Forsdyke, the specific-activity of the extracellular thymidine would only have been diluted by the intracellular thymidine pool and by any thymidine normally present in the medium. As well, the assay would only have measured the size of these thymidine pools. However, at thymidine concentrations above 5 μM, Sjöstrom and Forsdyke concluded that the most likely rate-limiting step was DNA polymerase. Therefore, the specific activity of the extracellular thymidine would have been diluted by the intracellular dTTP, dTDP, dTMP and TdR pools as well as by the *de novo* synthesis of thymidylate. Theoretically, the assay would then have measured the size of the total intracellular thymidine nucleotide and nucleoside pools.

When Adams used the method of Forsdyke to measure dTTP pools in cultured mouse fibroblast cells he indicated his understanding of the assumptions by stating that the method "... assumes that exogenous (thymidine) and endogenous dTTP are in direct equilibrium", but he did not justify or test this assumption (54).

In conclusion, the measurement of deoxyribonucleotide pools by the isotope-dilution assay is based on two untested - but testable - assumptions. Until these assumptions are proven, this method cannot be said to be a valid method for the measurement of deoxyribonucleotide concentrations in cells.

RADIOACTIVE ORTHOPHOSPHATE ASSAY

The first use of the ^{32}Pi method to measure deoxyribonucleotide pool sizes was by Neuhard and Munch-Petersen in 1966 (58). This method has not been used frequently and its use is declining, probably as a result of the increased use of more direct methods utilizing enzymatic or HPLC assays. The main assets of the ^{32}Pi method are that it is quite sensitive, conceptually and methodologically simple, and requires few assumptions. The main problems with this method are the long incubation times required for ^{32}Pi to equilibrate completely with the intracellular acid soluble phosphate compounds, and the difficulty in chromatographically separating the deoxyribonucleotides cleanly from other labeled compounds. It seems unlikely that further significant improvements can be made to this method.

General Principle:

The basic premise of this method is that if the pool of phosphate compounds in cells is equilibrated with external ^{32}Pi of known specific activity, then measurement of the amount of radioactivity in a given phosphate compound, such as dATP, will allow the calculation of the pool size of that compound.

The general procedure is that cells are labelled with ^{32}Pi of known specific activity until equilibrium is reached; that is until the specific activity of each phosphate acid-soluble phosphate compound equals the specific activity of the added ^{32}Pi. Attainment of equilibrium is variously taken as the time at which the total acid-soluble radioactivity becomes constant, the time at which the rate of incorporation of label into DNA becomes linear, or the time at which the amount of radioactivity in the compounds of interest becomes constant. The equilibration time should be determined for each treatment condition; i.e., for each drug, or for stationary or log phase cells, since equilibrium times can vary greatly from one condition to another (59). The cells are then extracted, and generally the extracts are treated with sodium periodate to oxidize the ribonucleotides, which are difficult to separate from the deoxyribonucleotides. The extracts are chromatographed and the amount of radioactivity in each deoxyribonucleotide is determined. These data plus the value for the specific activity of the ^{32}Pi allow the calculation of each pool size. Generally, only the triphosphates, the largest of the deoxyribonucleotide pools, can be determined accurately.

Development of the Assay:

The measurement of ribonucleotide pools by the ^{32}Pi method is relatively simple, but measurement of the very much smaller deoxyribonucleotide pools has been more difficult, mainly due to inability to separate the deoxyribonucleotides totally from the heavily labelled ribonucleotides and from ^{32}Pi. By 1965, procedures developed by Randerath and Randerath (60,61) and by Neuhard et al. (62)

allowed the separation of all eight ribo- and deoxyribonucleoside triphosphates using a two-dimensional chromatographic system on poly(ethylene)imine-cellulose thin layer plates. The first dimension solvent was 2 M LiCl: 2 N acetic acid (1:1 v/v) and the second dimension solvent was 3 M ammonium acetate in 5% boric acid, pH 7. In 1966, Neuhard and Munch-Petersen (58) and Neuhard (63) used this chromatographic system in conjunction with ^{32}Pi labeling to determine the size of the four deoxyribonucleoside triphosphate pools in E. coli 15T$^-$A$^-$U$^-$.

Colby and Edlin subsequently improved the chromatographic method of Neuhard et al. (62) in two ways: 1) Two concentrations of each solvent in each dimension were used, creating a concentration gradient and improving separations. 2) The cells were washed once with cold 0.15 M NaCl - 0.1 M Tris, pH 7.4 to remove excess ^{32}Pi before extraction and thereby reducing the streaking of ^{32}Pi (64). However, the washing procedure probably also caused nucleotide breakdown so that the net advantage of this step is questionable. Colby and Edlin also studied the kinetics of ^{32}Pi labeling of both the ribo- and deoxyribonucleoside triphosphate pools in growing, Rous sarcoma virus transformed, and growth-inhibited chick fibroblast cells (62). They found substantial differences in ^{32}Pi labeling kinetics among these cells, although the nucleotide pool sizes were similar. Although these were preliminary experiments, they demonstrated the need to determine the ^{32}Pi equilibration conditions under different growth conditions. Colby and Edlin also cautioned that their determinations of the deoxyribonucleoside triphosphates were quite variable due to ^{32}Pi streaking.

In 1971, Weber and Edlin confirmed the observation of Colby and Edlin regarding differences in the rate of ^{32}Pi uptake by growing and density-inhibited cells (59). They used a preliminary separation of inorganic and organic phosphates to reduce the extent of ^{32}Pi streaking during chromatography.

Probably the most significant improvements in the assay were introduced by Yegian in 1974 (65). He used periodate to oxidize the ribofuranosyl ring of the ribonucleotides, and thereby prevented the overlap of heavily labeled ribonucleotides with the deoxyribonucleotides during chromatography; this increased the accuracy of the determinations. This use of periodate oxidation to remove interference by ribonucleotides was not a new idea; in fact, Klenow had used it in 1962 to permit him to measure dATP concentrations using luciferase (40).

Yegian also introduced a new chromatography system that separated the four deoxyribonucleoside triphosphates much better than before, and eliminated interference from ^{32}Pi and the oxidized ribonucleotides. The main innovation was that samples were spotted in the middle of the plate and the ^{32}Pi was washed into the wick which was then cut off. The plate was then re-run with a different solvent in the opposite direction to separate the nucleotides. Finally, Yegian also checked the purity of each lot of ^{32}Pi, and used only lots that had less than 0.02% impurity. This precaution was not mentioned by previous researchers. In a review of this method, Hauschka noted that ^{32}Pi is often contaminated with polyphosphates and phosphosilicates, and these complicate the chromatographic purification of the nucleotides (1).

Bersier and Braun made additional changes in the method (66). They first measured the specific activity of the four ribonucleoside triphosphate pools directly by labeling large numbers of cells with ^{32}Pi for various times, extracting the cells, and isolating the ribonucleoside triphosphates by ion-exchange column chromatography. They found that in Physarum polycephalum, the ribonucleotide pools reached their maximum specific activity after 30 min of labelling; unfortunately they did not compare the specific activity of the ribonucleotide pools with that of the ^{32}Pi in the medium. This comparison would have been a test of the main assumption made by others that when the amount of label in a nucleotide pool becomes constant, the specific activity of each phosphate of the acid-soluble compounds equals the specific activity of the ^{32}Pi in the medium. Bersier and Braun then used the specific activities determined for the ribonucleotides plus the amount of label in each deoxyribonucleoside triphosphate, to calculate the deoxyribonucleoside triphosphate pool sizes. One other improvement they introduced was to measure nucleoside triphosphate recovery during extraction by adding tritiated nucleoside triphosphates to the extraction medium just before extracting the cells. They found that a considerable loss (60 to 70%) of the triphosphates had occurred, due mainly to triphosphate breakdown; the results were corrected for this loss during recovery.

Although improvements in the assay were being made, they were not always used. Thus in 1975 Nexo published a study of deoxyribonucleoside triphosphate concentrations in Tetrahymena pyriformis (62), using exactly the same procedures that Neuhard and Munch-Petersen had developed nine years earlier (58).

Finally, in 1977 Reynolds and Finch made further improvements (68). They found that the chromatographic method developed by Yegian did not completely remove all the oxidized ribonucleotides; some of these remained at the origin following washing, and radioactive orthophosphate was released during the chromatography, contaminating the deoxyribonucleotide spots. Reynolds and Finch overcame this by using the chromatographic system of Neuhard et al. (62) for periodate treated extracts. Streaking of orthophosphate occurred in the first dimension but in the second dimension, the overlapping orthophosphate ran ahead of the nucleotides, while any remaining orthophosphate that continued to leach from the origin ran parallel to, but away from the nucleotides.

No values have been published regarding the accuracy, reproducibility, or sensitivity of the ^{32}Pi deoxynucleoside triphosphate assay. However, in a few cases enough information has been given so that an estimate of the maximum sensitivity under those conditions can be made.

Bersier and Braun (66) found the equilibrium specific activity of the ribonucleotide pools in *Physarum* to be 13.5 counts/min per pmole; since the ^{32}Pi had equilibrated, the deoxyribonucleotide pools should have had the same specific activity. This value gives an indication of the sensitivity, but a value for the background radioactivity on the chromatograms would be necessary to complete the picture. Neuhard and Munch-Petersen reported the specific activity of the ^{32}Pi used to be 1-3 μCi/μmole, and theoretically this should have given equilibrium specific activities in the deoxyribonucleoside triphosphates of *E. coli* between 6.6 and 20 disintegrations/min per pmole (58). Again no value for the background radioactivity on the chromatogram was reported so the actual sensitivity cannot be determined. The use of higher specific activities would increase the sensitivity, but would also increase radiation damage to the cells. This is an important limitation on the sensitivity of this method.

Discussion:

The ^{32}Pi-deoxyribonucleotide assay has been in use for 14 years, undergoing constant evolution during this time. It is not possible to refer to a standard ^{32}Pi assay because almost every user of the assay has added some improvements; however, it is possible to examine all the variations and pick the best features of each. General features that should be included in a standard assay are as follows:

1) Purity checks of each batch of ^{32}Pi, followed by purification if there is significant contamination by polyphosphates or polysilicates which co-chromatograph with nucleotides.

2) Use of the highest ^{32}Pi specific activity which does not cause significant radiation damage, and of the lowest phosphate concentration in the medium which will still give normal growth rates.

3) Measurement of the ^{32}Pi equilibration times for each treatment condition. Attainment of equilibrium could reasonably be taken as the point at which the amount of radioactivity in the deoxyribonucleotide pools becomes constant.

4) Extraction of cells, with no prior washing, and neutralization on ice to minimize nucleotide breakdown. The extraction medium should contain a tritiated deoxyribonucleoside triphosphate for determination of overall recovery.

5) Oxidation of ribose compounds using sodium periodate, in order to improve chromatographic separation.

6) Use of a two-dimensional chromatography system such as that developed by Neuhard et al. (62), or Colby and

Edlin's modification of their system (64).

Although an assay incorporating these features would be the optimum ^{32}Pi deoxyribonucleoside triphosphate assay, it would still have the drawback of being more laborious than the enzymatic or HPLC assays.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The use of HPLC to measure deoxyribonucleoside triphosphates in cell extracts is a recent extension of the use of this technique to measure ribonucleotide concentrations. The HPLC method is direct, involves few assumptions, and allows the simultaneous measurement of all four deoxyribonucleoside triphosphates. It is less sensitive than the enzymatic assay, but offers approximately the same sensitivity as the ^{32}Pi assay. The HPLC assay is slower than the enzymatic assay when many samples must be processed, but it is better suited for a few samples, or for infrequent measurements. Since the HPLC assays in use are quite varied, there is a need to assess each one, to compare them to other methods, particularly the enzymatic assay, and to determine what further improvements should be made.

General Principle:

The HPLC assay relies mainly on three principles:

1) Separation: Deoxyribonucleotides can be rapidly separated using anion exchange columns and high performance chromatography apparatus. Separation from ribonucleotides

is facilitated by periodate oxidation of the ribonucleotides to change their mobilities.

2) Detection: Deoxyribonucleotides can be detected by measuring their uv absorbance. The ease and sensitivity of detection depends on the detector and the separation method. Solvents with relatively high refractive indexes or high uv absorbances, reduce the sensitivity of deoxyribonucleotide detection. As well, gradient separations generally result in a changing baseline due to changes in the refractive index or absorbance of the solvents. Finally, the sharpness of the peak will affect the accuracy of detection.

The amplification of the detector signal can usually be varied and a common measure of the detector sensitivity is the number of absorbance units required for full scale deflection of the recorder pen. For example, the Varian Aerograph LCS 1000 detector has a maximum sensitivity setting of 0.02 O.D. units full scale which may be equivalent to between 2000 and 3000 pmoles of dATP. Most new models, such as the Spectra-Physics 8000 HPLC detector and the Water Associates HPLC detector, have a maximum sensitivity setting of 0.005 O.D. units full scale which may be equivalent to between 500 and 750 pmoles of dATP. Although it is useful to know the detector sensitivity settings, these values can be misleading because the detectors are limited not only by how much the signal can be amplified, but also by the signal-to-noise ratio. Therefore, if this ratio is low, or if the separation method results in a shifting baseline, high sensitivity settings are of little value.

3) Quantitation. The amount of compound in a peak is proportional to its area on the recorder tracing. Other parameters such as peak height can be used for quantitative measurements, but except for very sharp, symmetrical peaks, they are not as sensitive or as accurate as measuring peak area. Peak areas are usually measured either by planimetry, which is accurate but tedious, or by automatic electronic integration. Although automatic integration is very convenient, it requires careful programming to ensure good accuracy and reproducibility.

Development of the Method:

The first published use of HPLC for the determination of deoxyribonucleoside triphosphate concentrations, was in 1978 (69,70). These reports were both from the same laboratory and the same method was used in each case. The separations were done isocratically on a Whatman Partisil SAX column (a strong anion exchange resin), using 0.45 M potassium phosphate buffer, pH 3.6. The peaks were monitored at both 254 and 280 nm, but the detector sensitivity settings were not reported. The only deoxyribonucleotides determined were dATP and dGTP. ATP and dATP were not separated at the base line, which was unstable thus making accurate integration of the peaks difficult; the method of integration was not given. Although dATP and dGTP values were also determined by the enzymatic method, the results obtained by the two methods were not compared.

The next obvious step was to get rid of interfering ribonucleotides using periodate oxidation. In 1979, this step was introduced by three laboratories (71-74), though two different procedures were used. Ritter and Bruce used the following procedure to oxidize the ribonucleotides: 200 µl 2 N formic acid and 200 µl sodium periodate solution (0.07 gm/ml) were added with mixing to 200 µl of cold neutralized PCA extract. After 45 min at 4°C in the dark, 200 µl of ethylene glycol was added to reduce unused periodate. After 15 min, 50 µl of concentrated ammonium hydroxide was added and 5 min later the pH was adjusted to 7.5. Although variable degrees of ribonucleotide oxidation were obtained with this procedure, they were satisfied with this method because as long as the ribonucleotide and deoxyribonucleotide concentrations were similar all could be separated. Separation was achieved using two 250 x 4.6 mm Whatman Partisil 10 SAX anion exchange columns in series, at 40°C, with a linear gradient from 0.2 M $NH_4H_2PO_4$, pH 3.2 to 0.6 M $NH_4H_2PO_4$, pH 4.4 at a flow rate of 1.0 ml/min for 2 h. The peaks were monitored at 254 nm, which is optimal for dATP but not for dCTP (λ_{max} = 280). The detector sensitivity was quite low (0.05 O.D. units full scale) and the peak areas were measured by planimetry (72).

This procedure has been tested in our laboratory using both standards and cell extracts with added tritiated ribo-

or deoxyribonucleoside triphosphates; it was found that the ribonucleotides were completely oxidized, and that the deoxyribonucleotides were completely stable (J.K. Lowe & J.F. Henderson, unpublished). Ritter and Bruce had concluded that the ribonucleotides were not always completely oxidized by this procedure and showed a chromatogram of a periodate treated cell extract in which there was a peak with the same retention time as UTP (72). Lowe and Henderson also noted this peak in periodate-treated cell extracts but not in treated standards. They concluded that the peak was not UTP since tritiated UTP added to a cell extract was completely oxidized, while this peak remained. The identity of this peak has not been established, nor is it known if it is produced during periodate treatment or if it normally co-chromatographs with UTP. Ritter and Bruce used two Partisil 10 SAX columns in series with a 2 hr linear gradient with column washing between runs (72), whereas Lowe and Henderson were able to obtain equally good resolution using a single Partisil 10 SAX column eluted isocratically with 0.25 M KH_2PO_4, 0.5 M KCl, pH 4.5, in ca. 40 min; no washing was required between runs (75).

The major problem with this oxidation procedure is the production of a very large and tailing peak of ribonucleotide oxidation products on whose shoulder the dCTP and dTTP peaks appear; this makes electronic integration virtually impossible and limits the accuracy of planimetric integration because of the difficulty in choosing a baseline.

Garrett and Santi solved the problem of the large tailing peak of ribonucleotide oxidation products by reacting them with methylamine to eliminate the phosphate groups and cleave the N-glycosidic bond (73). The resulting bases elute well before the deoxyribonucleoside triphosphates. The actual procedure of Garrett and Santi was as follows: to 1.0 ml of neutralized cell extract was added 40 µl 0.5 M $NaIO_4$, followed within several min by 50 µl of 4 M methylamine which had been slowly brought to pH 7.5 with H_3PO_4. After 30 min at 37°C, 10 µl of 1 M rhamnose was added to reduce any remaining periodate. The oxidized samples were chromatographed on a Whatman Partisil-10 SAX anion exchange column (4.6 x 250 mm) and eluted isocratically with 0.4 M ammonium phosphate pH 3.25: acetonitrile (10:1) at 30°C. The peaks were monitored at 254 nm at 0.013 O.D. units full scale. Peaks of less than 500 pmoles could not be reliably integrated with an automatic electronic integrator and were quantitated by comparing the height with that of standard peaks (rather than by planimetry which would have probably been more accurate because the peaks were asymetric). They reported a lower limit of sensitivity of 30 pmoles. Values for accuracy and reproducibility were not given.

Lowe and Henderson used this method both for deoxyribonucleoside triphosphate standards and for neutralized cell extracts (unpublished results). After periodate and methylamine treatment the extracts were chromatographed on a Whatman Partisil 10 SAX anion exchange column and eluted isocratically with either 0.4 M ammonium phosphate, pH 3.25: acetonitrile (10:1) or 0.25 M KH_2PO_4, 0.5 M KCl, pH 4.5; equivalent results were obtained with either solvent. The peaks were detected at 254 nm, at a sensitivity of 0.02 or 0.005 O.D. units full scale, and were measured by planimetry rather than electronically because of an unstable baseline. In agreement with Garrett and Santi (73), Lowe and Henderson found that the methylamine treatment greatly reduced the tailing of the initial peak, making the dTTP and dCTP peak measurements easier than in cell extracts oxidized by the method of Ritter and Bruce (72). Although peaks containing as few as 30 pmoles were visible, it was impossible to obtain consistent values for the areas because the position of the baseline could not be accurately determined. Although Garrett and Santi reported that 30 pmoles of deoxyribonucleotide could be measured by this method, Lowe and Henderson found this value to be ca. 100 to 200 pmoles. This difference may simply reflect the different machines used by the two groups. Garrett and Santi used a Hewlett-Packard HPLC while Lowe and Henderson used both a Varian and a Water Associates HPLC.

The most recent changes to the method have been made by Maybaum _et al_. (75). Their method involves the separation and collection of each ribo- and corresponding deoxyribonucleotide using an anion exchange column. Each pair of nucleotides is converted to the corresponding nucleoside by acid phosphatase, and then separated and collected using a preparative reverse-phase column. When higher sensitivity is required, the fractions are rerun on an analytical reversed phase or cation-exchange column. Thus they are able to measure the eight major ribo- and deoxyribonucleotides, although the assay will be described and discussed only in the context of the deoxyribonucleotide measurements. The published procedure is restricted to pyrimidines but an unpublished modification of the procedure has been developed for purines.

The actual procedure used by Maybaum *et al*. was as follows: neutralized cell or tissue extracts were chromatographed on an analytical anion exchange column. Monophosphates were eluted isocratically followed by a linear gradient to elute the di- and triphosphates. Ribonucleotides were not separated from the corresponding deoxyribonucleotides, so each pair was collected, lyophilized and treated with acid phosphatase to convert the nucleotides to nucleosides. Each pair of ribo- and deoxyribonucleosides were run on a preparative reverse-phase column because the high salt content would saturate an analytical column. Uracil and cytosine nucleosides were eluted with methanol: H_2O and thymidine was eluted with acetonitrile: H_2O. Detection was at 254 and 280 nm with the lower limit of sensitivity at 100 pmoles and a net recovery of 80%.

In order for this method to be of practical use in measuring deoxyribonucleotides in cell extracts, much higher sensitivity was required. Therefore, each deoxyribonucleoside was collected, dried, and redissolved. Deoxyuridine and deoxythymidine were rechromatographed on a reverse-phase column and eluted with 10 mM NaH_2PO_4, pH 3.0: acetonitrile and 10 mM sodium acetate, pH 7.4: acetonitrile, respectively, while deoxycytidine was rechromatographed on a cation exchange column and eluted isocratically. With detection at 254 and 280 nm, the lower limit of sensitivity was 10 pmoles and the net recovery of the entire procedure was 66%. The standard deviation, based on three determinations for each deoxyribonucleotide ranged from 3 to 15%.

Although this procedure can provide a considerable amount of information from one sample, it is very tedious and time consuming, with only a 3-fold more sensitive measurement of the deoxyribonucleoside triphosphates over that obtained by Garrett and Santi (73).

Discussion

HPLC has been used to assay deoxyribonucleoside triphosphates for only four years, but it is quite useful within certain limitations of sensitivity, speed and cost. Although Maybaum et al. were able to measure amounts of deoxynucleotides as low as 10 pmoles, an almost prohibitive amount of work is required (75). Their method would not be practical for analyzing more than a few samples.

There are two main ways the sensitivity of the HPLC method could be enhanced: 1) the development of new columns with higher theoretical plates, and 2) the development of new detectors with a higher signal-to-noise ratio than is presently available.

Given the limitations of the present equipment, the HPLC assay is still useful if a large number of cells can be extracted or if the deoxyribonucleotide pool to be measured is artificially elevated. For example, the dATP pool in red blood cells incubated with deoxyadenosine plus 2'-deoxycoformycin is sufficiently elevated to allow it to be easily measured using HPLC (C.M. Smith and J.F. Henderson, unpublished).

ENZYMATIC ASSAY

The enzymatic assay of deoxyribonucleoside triphosphates was developed by Solter and Handschumacher in 1969 (16). Many subsequent improvements and modifications have resulted in a plethora of assays. Presently there are more than twenty different variations of this method in the literature, many of which have not been thoroughly evaluated, and some of which certainly are not optimal. As a result it is difficult to compare data obtained using different variations of the method, without having an assessment of the reliability of the different methods, and as well, researchers wishing to use the enzymatic assay are faced with a difficult choice. Finally, there are improvements which would benefit even the best of the present assays. The purpose of this review is to describe the development of the enzymatic assay, to present both the advantages and the drawbacks of each variation of the assay, and finally to present the method used in our laboratory, into which we have attempted to incorporate the best features of previous assays as well as further improvements.

General Principle:

The enzymatic assay is based on the fact that DNA polymerase I will accurately catalyze the incorporation of four deoxyribonucleoside triphosphates into DNA, which serves both as the template and the primer for the reaction. If three of these triphosphates are present in excess, and one of the three is radioactive, then at the completion of the polymerization reaction the amount of radioactivity in DNA will be proportional to the amount of the fourth, limiting triphosphate that was originally present. One of the important improvements to the original assay was the later substitution of synthetic alternating co-polymers for the DNA.

An important requirement of the assay is that in order for the optimum incubation time of the reaction to be independent of the amount of limiting deoxyribonucleotide present, the kinetics of the polymerization reaction must be first-order with respect to the limiting deoxyribonucleotide. Thus, maximum incorporation will be reached at the same time regardless of the amount of deoxyribonucleotide being assayed.

Development of the Assay

The first enzymatic assay for deoxynucleoside triphosphates used undenatured calf thymus DNA as the template-primer and *E. coli* DNA polymerase I as the polymerization enzyme; deoxynucleoside triphosphate pools in extracts of bacterial and mammalian cells were measured (16). Time courses demonstrated that the reaction was first order with respect to the limiting substrate and also that the product was degraded with time as a result of 3'→5' exonuclease activity, an inherent property of the *E. coli* polymerase I. The assay was quite insensitive by modern standards, with a lower limit of 100 pmole/tube, although the authors pointed out that the sensitivity could be increased by increasing the specific activity of the labeled deoxyribonucleotides. By assaying the extracts in the presence and absence of standards, Solter and Handschumacher demonstrated that cell extracts did not affect the accuracy of the assay.

In 1970, Skoog (7) and Lindberg and Skoog (8) introduced a significant improvement in the assay by using poly[d(IC)] as the primer-template for the dGTP and dCTP assays, and poly[d(AT)] for the dATP and dTTP assays. The synthetic alternating copolymers offered several advantages over DNA in that they were readily soluble, resulted in a faster polymerization rate, and because of their alternating deoxy-nucleotide sequence, they incorporated labeled and unlabeled nucleotide in exactly equal amounts and required only two substrates for the reaction. By also using nucleotides with higher specific activities than did Solter and Handschumacher, Lindberg and Skoog reported a lower limit of sensitivity of 0.2 pmoles. Unlike Solter and Handschumacher, Lindberg and Skoog tested for and found phosphatase activity in the DNA polymerase I preparation; they were able to inhibit this by pretreating the polymerase with mercuric ion.

The next significant improvement was added by Munch-Petersen _et_ _al_. who recognized that the specific activities of the labelled deoxyribonucleotides were being diluted by the same unlabelled deoxyribonucleotides in the cell extract (10). For example, an increase in the amount of dATP in the cell extracts would lower the apparent concentration of dTTP by diluting the specific activity of the [^{3}H]dATP in the assay. To correct the data for this effect, they constructed appropriate simultaneous equations.

Between 1973 and 1980 few if any improvements were made to the assay. Our own recent improvements involve the use of a 3'→5' exonuclease inhibitor to increase the stability of the product polymer. The other improvement is the result of the discovery that background incorporation can occur in the presence of the labeled deoxyribonucleotide alone, presumably as the result of terminal addition of the labeled nucleotide onto the primer-template. When the specific activity of the label is increased in order to increase the sensitivity and accuracy of the assay, the background incorporation increases proportionally. Thus achievement of greater sensitivity and accuracy requires that the background incorporation be corrected for the dilution of the specific activity of the radioactive precursor by unlabeled nucleotide in the cell extract.

One final improvement, which probably would not increase the accuracy or sensitivity but which would make the assay less tedious, would be the use of a DNA polymerase with no associated 3'→5' exonuclease activity, such as calf thymus DNA polymerase. This would eliminate the need for an exonuclease inhibitor, which is not completely effective, and would also eliminate the need for precise sampling times. Our attempts to use a commercial calf thymus DNA polymerase preparation in this assay were unsuccessful because the preparation was contaminated both with phosphatase and exonuclease activities.

Important Variables in Individual Methods:

More than 20 variant DNA polymerase assays for deoxyribonucleoside triphosphates have been published. The important differences among them will be described in the following section and in Tables 2 to 5.

DNA polymerase. Three types of DNA polymerase have been used in the enzymatic assay: *E. coli* polymerase I, *Micrococcus luteus* polymerase I, and the large fragment of *E. coli* polymerase I (Table 2). The *E. coli* and *M.*

Table 2

DNA polymerase preparations used

Type of polymerase	Specific Activity (units/mg protein)	Phosphatase detected	Reference
E. coli, Pol I	8,900	Yes	7
E. coli, Pol I	18,000	b	8
E. coli, Pol I	a	b	9
M. luteus, Pol	1,336	Yes	3
E. coli, Pol I, large fragment	a	b	2
E. coli, Pol I, large fragment	a	b	10
M. luteus, Pol	600	b	11
M. luteus, Pol	a	b	12
M. luteus, Pol	a	b	13
M. luteus, Pol	a	b	14
E. coli, Pol I, large fragment	a	b	15
E. coli, Pol I, large fragment	7,050	b	16
E. coli, Pol I, large fragment	a	b	17
M. luteus, Pol	a	b	18

(... cont'd.. Table 2 ...)

Type of polymerase	Specific Activity (units/mg protein)	Phosphatase detected	Reference
M. luteus, Pol	420	b	19
E. coli, Pol I	a	b	20
E. coli, Pol I	59,000	b	21
M. luteus, Pol	a	b	22
E. coli, Pol I	22	b	23
M. luteus, Pol	10,000	b	24
E. coli, Pol I	4,600	b	25
M. luteus, Pol	a	b	26
M. luteus, Pol	a	b	27
E. coli, Pol I	3,600	b	c

a) not reported

b) not determined

c) D. Hunting and J.F. Henderson, unpublished; see Appendix.

luteus polymerases are very similar and have the same types of activities, while the large fragment of *E. coli* polymerase I contains 5'→3' polymerase and 3'→5' exonuclease but not 5'→3' exonuclease activity (77).

The main problem with DNA polymerase preparations is that they often contain contaminating phosphatase activity which at the very least reduces the sensitivity of the assay by dephosphorylating the substrates, thus making them unavailable for DNA synthesis. We have found phosphatase activity in DNA polymerase preparations from several sources, but have been able to obtain phosphatase-free preparations from Boehringer-Mannheim Inc. (D. Hunting and J.F. Henderson, unpulished; see Appendix). Although it is useful to know the specific activity of the enzyme preparation, it is more important to test for the presence of phosphatase, even in preparations of high specific activity. Table 2 shows that even a polymerase preparation of high specific activity contained significant amounts of phosphatase activity. Unfortunately, phosphatase activity was only assayed in three of the twenty-four variations of the assay.

Reaction conditions. The polymerases used in this assay all contain 3'→5' exonuclease activity; therefore, when the polymerization reaction has gone to completion, degradation of the product begins, releasing labelled deoxyribonucleotides. The presence of this exonuclease activity makes it essential to optimize the incubation time. We use an exonuclease inhibitor (dAMP) to stabilize the product polymer and reduce the error caused by small changes in sampling times, the rate of polymerization, or 3'→5' exonucleation. Even in the presence of an exonuclease inhibitor some exonucleation occurs, so optimization of the sampling time still is important. The actual value for the sampling time is probably not too important; however excessively long incubations at 37°C may result in triphosphate breakdown and should be avoided.

The amount of primer-template used per tube is important for two reasons. There should be sufficient primer-template to make the polymerization reaction independent of its concentration, but since background incorporation is proportional to the amount of primer-template, excessive amounts should not be used. Unfortunately, it is difficult to compare the amounts of primer-template used in the various assays because different units were used. In addition, the properties of primer-templates from different sources vary. For example, we have found that poly[d(IC)] purchased from the Sigma Chemical Co. had much less primer-template activity per A_{260} unit than did a similar polymer obtained from the Miles Chemical Co. (D. Hunting and J.F. Henderson, unpublished). Information concerning the relative sizes of the polymers was not available. As well, in some assays the DNA in primer-template was pretreated by denaturation or treatment with DNase I to increase the activity (19,21).

As discussed earlier, the synthetic polymers are better suited to the enzymatic assay than is DNA. If DNA is used it would be wise to ensure that the labelled nucleotide is complementary to the nucleotide being assayed, so that one can be sure they will be incorporated in equal amounts.

Table 3 compares the reaction conditions used in the various enzymatic assays. Sampling time was optimized in only 50% of the assays. Some of the sampling times were quite long (e.g., 120 min, 360 min), but most were between 30 and 60 min. In a few assays the amount of primer-template used seems to have been excessive, thereby increasing the background incorporation. We have found 0.02 A_{260} units of either poly[d(AT)] or poly[d(IC)] to be sufficient in our system. In many cases in which DNA was the primer-template, the labelled nucleotide and the nucleotide being assayed were not complementary; often the same labeled nucleotide was used to determine the concentrations of the other three nucleotides, so that the labeled and unknown nucleotides were complementary in only one of the assays.

Controls. There are a number of controls which need to be performed on the assay before using it routinely.

First, it is important to measure the purity of the non-radioactive nucleotides which are used as standards in the assay in order that the true concentration of the deoxyribonucleotides can be determined, rather than just

Table 3

Reaction Conditions

Sampling time optimized	Sampling time (min)	Primer-template per tube			Label and unknown complementary	Reference
		poly[d(AT)]	poly[d(IC)]	DNA		
Yes	40	-	0.6 nmoles		Yes	7
Yes	35	4 nmoles	-		Yes	8
Yes	35-40	4 nmoles	0.6 nmoles		Yes	9
Yes	25	0.1 μg	0.1 μg		Yes	3
Yes	30-40 or 80-120	-	0.2-0.8 nmoles		Yes	2
Yes	30-40	10 nmoles	-		Yes	10
No	60	4-23 A_{260} U	-		Yes	11
No	30	0.01 A_{260} U	-	a	No	12
Yes	60	5 nmoles	5 nmoles		Yes	13
No	35	0.05 A_{260} U	-	5 μg	No	14
No	40		0.6 nmoles		Yes	15
Yes	60	-	-	10 μg	Not always	16
Yes	25	-	-	10 μg	Not always	17

(... cont'd .. Table 3 ...)

Sampling time optimized	Sampling time (min)	Primer-template per tube			Label and unknown complementary	Reference
		poly[d(AT)]	poly[d(IC)]	DNA		
Yes	120	-	-	10 μg	Not always	18
No	45	-	-	1.5 μg	Not always	19
No	60	-	-	10 μg	Yes	20
No	45	-	-	12 μg	Not always	21
No	60	-	-	5 μg	Not always	22
No	30	-	-	10 μg	Not always	23
No	360	-	-	7.5 μg	No	24
No	90	-	-	7.5 μg	No	25
Yes	90	-	-	5 μg	Not always	26
No	a	-	-	10 μg	Not always	27
Yes	10-20 or 40-60	0.02 A_{260} U	0.02 A_{260} U	-	Yes	b

a) not reported.

b) D. Hunting and J.F. Henderson, unpublished; see Appendix.

relative values. We have found, for example, that the deoxyribonucleoside triphosphates purchased from the Sigma Chemical Co. and shipped at ambient temperatures contained ca. 2 to 10 molar % of the mono- and diphosphates, as determined by HPLC. Triphosphates shipped on dry-ice contained only ca. 2 molar % of the mono- and diphosphates upon arrival, but even storage at -20°C over a dessicant for a few months resulted in as much as 15% hydrolysis of the triphosphates. dGTP was the least stable but substantial hydrolysis of all the triphosphates occurred. Maximum stability was achieved by preparing stock solutions of the triphosphates in buffer followed by storage at -20°C. These solutions were stable for at least several months.

In addition, if the data are to be corrected for dilution of the specific activity of the radioactive nucleotide by the extract, then the purity and specific activity of the radioactive deoxyribonucleotides must be known. We have found that in tritiated deoxyribonucleoside triphosphates preparations supplied by ICN and by Schwartz-Mann Inc., 6-14% of the radioactivity in the nucleotides was present as mono- and diphosphates as determined by thin-layer chromatography. As well, substantial amounts (up to 50%) of the radioactivity in some preparations was present on arrival as tritiated water. Therefore it is important to determine the amount of hydrolysis of the radioactive triphosphate and the amount of exchange of the tritium that has occurred.

Finally, the effect of the cell extract on the assay must be determined for each different type of extract and each type of experimental condition used. This can be done in three ways: 1) by addition of deoxyribonucleoside triphosphate standards to the cell extracts to determine if the final amount measured is equal to the sum of the amounts in the standard and cell extract; 2) by determining if the values obtained from the assay are proportional to the amount of cell extract assayed; and 3) by performing time courses to determine if the assays containing standards, control cell extracts, and extracts of drug treated cells, reach the maximum incorporation at the same time.

These controls on the effect of the extract on the assay are very important. As mentioned in the discussion of extraction methods, North et al. have reported that methanol extracts of cells gave artificially high deoxyribonucleoside triphosphate values (5). They also reported that PCA extracts of cells interfered slightly with the assay procedure, although this is not consistent with our results (D. Hunting and J.F. Henderson, unpublished) or the results of many other researchers (3,8,13,16,17,19,20, 21,24,26). As well, Tyrsted has reported that 60% methanol extracts of unwashed cells interfered with the enzymatic assay of dCTP (2).

Table 4 presents the results of a comparison of the

Table 4

Controls Performed

Purity of unlabelled nucleotides measured	Purity of radioactive nucleotides measured	Effect of cell extract on assay measured	Reference
Yes	Yes	Yes	7
Yes	Yes	Yes	8
Yes	Yes	Yes	9
No	No	Yes	3
No	No	Yes	2
No	No	Yes	10
No	No	No	11
No	No	No	12
Yes	Yes	Yes	13
No	No	Yes	14
No	No	No	15
No	No	Yes	16
No	No	Yes	17
No	No	No	18

(... cont'd .. Table 4 ...)

Purity of unlabelled nucleotides measured	Purity of radioactive nucleotides measured	Effect of cell extract on assay measured	Reference
No	No	Yes	19
No	No	Yes	20
Yes	No	Yes	21
No	No	No	22
No	No	No	23
No	No	Yes	24
Yes	Yes	Yes	25
No	No	Yes	26
No	No	No	27
Yes	Yes	Yes	a

a) D. Hunting and J.F. Henderson, unpublished; see Appendix.

controls performed on the different assays. In most cases, the purity of neither the labeled nor unlabeled nucleotides was determined, making it impossible to correct the results for dilution of the labeled nucleotide by the extract. This correction was made in only five of the twenty-four assays (7,8,9,13,25, D. Hunting and J.F. Henderson, unpublished), even though the importance of this correction was demonstrated by Munch-Petersen _et al_. in 1973 (10). In a few cases, however, this correction would not have been too important because a large excess of radioisotope was used, hence little dilution would have been caused by the sample.

Our own assay procedure is the only one which includes a correction for the effects of dilution of the specific activity of the labeled nucleotide on background incorporation. This correction is required only when radioactive nucleotides of high specific activity are used or when small amounts of nucleotides are measured.

Only seven of the twenty-four methods did not include a check on the effect of the cell extract on the assay.

Sensitivity, Reproducibility, Accuracy and Range: These parameters are all important, but in fact only reproducibility and accuracy are measures of the quality of a particular method. Often, the sensitivity and range used are simply determined by the type of samples that are to be analyzed. Table 5 compares the sensitivity, reproducibility

Table 5

Sensitivity, Reproducibility and Range

Sensitivity $\left(\frac{\text{cpm incorporated}}{\text{pmole substrate}}\right)$	Reproducibility	Range (pmoles)	Reference
150-900	a	0.2-4	7
700-1,000	a	0.5-7	8
a	a	a	9
700-1,200	S.D. = 0.1	0.2-40	3
300-600	a	0-6	2
350	a	1-10	10
1,600	a	0.1-5	11
a	a	1-100	12
a	S.E.M.<±7%	a	13
a	C.O.V.=1.2%	0-50	14
a	a	a	15
1-3	a	100-1,000	16
a	a	1-20	17
a	a	2-50	18

(... cont'd .. Table 5 ...)

Sensitivity ($\frac{\text{cpm incorporated}}{\text{pmole substrate}}$)	Reproducibility	Range (pmoles)	Reference
a	a	0-100	19
a	a	a	20
a	a	5-100	21
a	a	a	22
a	a	5-500	23
a	a	0-100	24
4	a	0-200	25
a	a	a	26
a	a	a	27
500-5,000	S.D. = 2.5-8.5%	0.5-200	b

a) not reported.

b) D. Hunting and J.F. Henderson, unpublished; see Appendix.

and range of the various assays. Accuracy values were available for only one of the assays.

The sensitivity is expressed as the number of counts per minute incorporated into the polymer for each pmole of limiting substrate. In most cases these values were calculated from standard curves presented in the literature. Values for sensitivity in pmoles per tube were generally not available. It is obvious that the enzymatic assay is capable of very high sensitivity since the radioactive deoxyribonucleoside triphosphates are available at very high specific activities, such as 20 Ci/mmole. Although values for reproducibility are rarely presented, those given are good. It is also apparent that the enzymatic assay can be used over a very wide range (about 10,000-fold) of concentrations.

Conclusions. Although in principle the enzymatic assay of deoxyribonucleoside triphosphate concentrations is capable of very good sensitivity, accuracy, and reproducibility, in practice it is often not used to its full potential. Several improvements to the assay, such as the use of synthetic copolymers, the tests for phosphatase activity in the DNA polymerase preparation, and the correction of the results for the dilution of the radioactive precursor by non-radioactive precursor in the sample, are still not incorporated into all the versions of the assay. Furthermore, several versions of the assay have not been thoroughly tested to determine if they measure the actual deoxyribonucleoside triphosphate concentrations in cell extracts. It is hoped that this review will alert researchers to the possible pitfalls in this method as well as to its potential.

CONCLUSIONS

There are three useful methods for measuring deoxyribonucleoside triphosphate concentrations in cells at the present time: the ^{32}Pi, HPLC and enzymatic assays. The microbiological and chromatographic methods are too insensitive to be useful unless large amounts of biological material are available. Table 6 gives the approximate maximum sensitivity that has been achieved with each method, and as well gives the main limitations on the sensitivity of each method. All of the methods can be reliable and accurate when used properly. The HPLC assay has the advantage of requiring less time to set-up than the enzymatic or ^{32}Pi assays, which require more control experiments. Therefore, if few or infrequent measurements are to be made the HPLC method may be best suited. The enzymatic method has the advantages of being ca. 100-fold more sensitive than the ^{32}Pi or HPLC assays, as well as faster when large numbers of samples are involved. The ^{32}Pi assay has no apparent advantages over the HPLC or enzymatic assays, since it is slower than both and less sensitive than the latter. The

Table 6

Sensitivities of different deoxyribonucleoside triphosphate assay methods

Assay method	Maximum sensitivity achieved (pmoles/assay)	Factors limiting sensitivity	References
Microbiological	500	Growth response of organism to deoxyribonucleosides; volume required for determination of growth rate.	30
Chromatography with uv measurement	<400[a]	Sensitivity of uv measurement; background absorbance.	42
Radioactive orthophosphate (^{32}Pi)	10[b]	Sensitivity of organism to radiation; background radioactivity on chromatogram.	58
HPLC with uv detection	10	Sensitivity of uv detection; background absorbance.	76
Enzymatic	0.1	Specific activity of deoxyribonucleoside triphosphates; washing background due to incomplete removal of unincorporated radioactive triphosphates.	3,7,11

Table 6 Footnotes

[a] This value is based on the fact that Kummer and Kraml extracted 1 x 10^7 cells containing 400 pmoles of dTTP. However since they did not report the amount of extract that was chromatographed the value given is a minimum estimate of the sensitivity of the method.

[b] This value is based on an intracellular deoxyribonucleoside triphosphate specific activity of 20 dpm/pmole and the assumption that 200 dpm can be accurately determined.

final choice of methods is probably between the HPLC and enzymatic assays, and this choice will depend on the particular requirements of the researcher and the equipment that is available.

Finally, when comparing intracellular deoxyribonucleotide concentrations given in the literature, one must assess not only the general assay method, but also the particular variant method that was used.

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APPENDIX I

Improved Enzymatic Assay

The measurement of intracellular deoxyribonucleoside triphosphates by the enzymatic method as used in our laboratory is described here. This procedure incorporates the best features of other assay procedures, as well as improvements of our own, as discussed above.

Cell Extraction: Preparation of extracts for nucleotide pool size measurements were as follows: $0.25\text{-}4.0 \times 10^7$ cells were centrifuged at 1000 g for 2 min at 4°C. The medium was aspirated and the tube recentrifuged at 1000 g for 5 sec to remove medium from the centrifuge tube wall. The pellet was extracted on ice with 0.4 M PCA containing $[^3H]$adenosine for determination of dilution. After 30 min the extract was centrifuged and the supernatant was removed and neutralized by extraction with 0.5 M alamine 336 (tricapryl tertiaryamine) in Freon-TF (trichlorotrifluoroethane) (77). Supernatants were stored at -20°C. Analysis by HPLC of samples stored for several weeks showed no nucleotide breakdown.

Purity of Reagents: The purities of the non-radioactive deoxyribonucleoside triphosphates were 90-98 molar % as determined by HPLC. The final concentration of each deoxyribonucleoside triphosphate was corrected for the presence of the impurities which were deoxyribonucleoside mono- and diphosphates. The standard nucleotide solutions were stable for several months at -20°C.

The radioactive deoxyribonucleoside triphosphates were supplied and stored in 50% ethanol. The ethanol and tritiated water were removed by lyophilization followed by dissolution in 100 mM Hepes, pH 7.4. The radiochemical purity was 86 to 94% as determined by chromatography followed by sample oxidation and liquid scintillation counting. The solution was stable for several months at -20°C.

DNA polymerase I (_E. coli_) was supplied and stored in 50% glycerol, pH 7.0. A working solution was prepared by diluting the stock solution with 50 mM Tris-HCl, pH 7.8 containing 12 mg/ml bovine serum albumin. This solution was stored not longer than one month. Each new batch was checked for phosphatase activity by incubating the enzyme with all the components of the assay except the copolymer. The formation of deoxyribonucleoside mono- and diphosphates indicated the presence of phosphatase activity. All the enzyme preparations used in the assay were phosphatase-free.

Reaction Conditons: The following components were common to both the dATP and dTTP assay in a final volume of 180 µl: 0.02 A_{260} units poly[d(AT)], 1.8 µmoles $MgCl_2$, 1.8 µmoles dAMP, 18 µmoles Hepes buffer, pH 7.4, and 0.75 Richardson units of DNA polymerase I (78). As well, the dATP assay contained 100 pmoles (0.5 µCi) $[^3H]$dTTP, and 0 to 75 pmoles dATP standard, while the dTTP assay contained 100 pmoles (0.5 µCi) $[^3H]$dATP and 0 to 75 pmoles dTTP standard.

The following components were common to both the dGTP and the dCTP assays in a final volume of 180 µl: 0.02 A_{260}

units poly[d(IC)], 1.8 μmoles $MgCl_2$, 1.8 μmoles dAMP, and 18 μmoles Hepes buffer, pH 7.4. As well, the dGTP assay contained 100 pmoles (2.2 μCi) [^{3}H]dCTP, 0 to 10 pmoles dGTP standard and 1.9 units DNA polymerase I. The dCTP assay contained 240 pmoles (0.5 μCi) [^{3}H]dGTP, 0 to 200 pmoles dCTP standard and 3.0 units DNA polymerase I.

The dAMP was used to inhibit the 3' to 5' exonuclease activity of the DNA polymerase I (79).

The reaction was started by the addition of the DNA polymerase I, followed by incubation at 37°C. At each time point aliquots were removed and spotted on squares of Whatman 3MM filter paper which had been wetted with 200 μl of 2% sodium pyrophosphate. The squares were washed (3 x 15 min) with a solution of 5% TCA and 1% sodium pyrophosphate (20 ml/square), then rinsed once with 95% ethanol and finally washed (1 x 15 min) with 95% ethanol. The dried filters were counted in toluene scintillation cocktail (4 gm PPO and 0.1 gm POPOP per litre of toluene).

Results were corrected for the washing background and for the effects of the dilution of the specific activity of the labeled deoxyribonucleotide by the sample on both the sample incorporation and the background incorporation.

The final value for the deoxynucleoside triphosphate concentration was independent of the amount of the extract used in the assay, within the limits of the standard curves. As well, addition of standards to cell extracts were used to demonstrate that the assay was not affected by the cell extracts. Finally, time courses were performed with standards, cell extracts of control cells, and cell extracts of drug treated cells to ensure that the maximum incorporation was reached at the same time under all conditions.

Correction of the data for the isotopic-dilution caused by the sample: The samples being assayed usually contain nonradioactive deoxyribonucleoside triphosphate which will dilute the specific activity of the radioactive nucleotide used in the assay. As a result, both the background incorporation and the sample incorporation will be lowered, in proportion to the amount of isotopic dilution that has occurred.

The following derivation provides the equations to correct the incorporation data for the isotopic-dilution caused by the sample.

Definitions:

dNTP1 and dNTP2:	deoxyribonucleoside triphosphate 1 and 2 which are incorporated into the alternating copolymer.
P1:	pmoles of radioactive dNTP2 per tube in the dNTP1 assay.
P2:	pmoles of radioactive dNTP1 per tube in the dNTP2 assay.
d1 and d2:	dilution of the radioactive nucleotide caused by the sample in the dNTP1 and dNTP2 assays, respectively.

C1 and C2: Actual radioactivity incorporated into the polymer in the dNTP1 and dNTP2 assays, respectively.

Y1 and Y2: Dilution-corrected radioactivity incorporated into the polymer.

m1 and m2: slope of the standard curve in each assay.

X1 and X2: pmoles of dNTP1 and dNTP2 respectively in the sample or standard, per tube.

b1 and b2: Background incorporation in the assays, i.e., the Y-intercepts of the standard curves.

V1 and V2: Volumes of sample used in each of the assays.

Since the standard curves are linear the following relationships can be defined:

1) $Y1 = m1X1 + b1$

2) $Y2 = m2X2 + b2$

But the dilution-corrected cpm (Y) is equal to the actual radioactivity incorporated (C) divided by the isotopic-dilution caused by the sample (d).

3) $Y1 = \frac{C1}{d1}$

4) $Y2 = \frac{C2}{d2}$

The isotopic-dilution, when corrected for the volume of sample used in each tube, can be expressed as:

5) $d1 = \frac{P1}{P1 + \frac{X2V1}{V2}} = \frac{P1V2}{P1V2 + X2V1}$

$$6)\ d2 = \frac{P2}{P2 + \frac{X1V2}{V1}} = \frac{P2V1}{P2V1 + X1V2}$$

These equations can be solved for X1 and X2:

$$X1 = \frac{(C1P1P2m2V2 + C1C2P2V1 - P2C1b2V1 - b1P1P2m2V2)}{V2(P1P2m1m2 - C1C2)}$$

$$X2 = \frac{(C2P1P2m1V1 + C1C2P1V2 - P1C2b1V2 - b2P1P2m1V1)}{V1(P1P2m1m2 - C1C2)}$$

We have incorporated these equations into a computer program, written in APL, which also includes linear regression analysis. The program determines the slopes and Y-intercepts of the standard curves, and then uses this information in the solution of the equations for X1 and X2.

# CHAPTER 3

DETERMINATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATES USING DNA POLYMERASE: A CRITICAL EVALUATION

INTRODUCTION

The assay of deoxyribonucleoside triphosphate concentrations by the use of DNA polymerase (EC 2.7.7.7) was developed in 1969 by Solter and Handschumacher (1). This method is based on the fact that DNA polymerase I will catalyze the incorporation of four deoxyribonucleoside triphosphates into DNA. If three of the triphosphates are present in excess, and one of the three is radioactive, then the amount of radioactivity incorporated into the DNA will be proportional to the amount of the fourth, limiting triphosphate, that is present. More recently, the method has been improved by the substitution of poly[d(AT)] and poly[d(IC)] for DNA (2,3), but the basic principle remains unchanged.

Other improvements (e.g., correction for dilution of the specific activity of added radioactive deoxyribonuleoside triphosphates by the deoxyribonucleoside triphosphates to be assayed (4,5)) have also been made, and other variations have been introduced by those using the method; there now are more than 20 appreciably different assays using DNA polymerase in the literature. A number of the changes that have been made have not been critically evaluated, and factors that should be checked in any use of this assay have not always been considered. The purpose of the present study was to critically evaluate each aspect of the assay and thereby to develop a reliable method for the determination of intracellular concentrations of dTTP, dCTP, dATP and dGTP.

MATERIALS AND METHODS

Chemicals. [8-^{3}H]dATP (10 Ci/mmol), [8-^{3}H]dGTP (19 Ci/mmol), [5-^{3}H]dCTP (20 Ci/mmol), [methyl-^{3}H]dTTP (17 Ci/mmol), and [8-^{3}H]adenosine (11 Ci/mmol) were purchased from Schwarz/Mann Corp. dATP, dGTP, dCTP, dTTP and poly d(AT) were purchased from Sigma Chemical Co. Poly d(IC) was purchased from Miles Chemical Co. DNA polymerase I, (1500-5000 units/mg) from *E. coli* was purchased from Boehringer Mannheim Corp. Alpha MEM medium and dialyzed fetal calf serum were purchased from Grand Island Biological Co.

Cells. Chinese hamster ovary-K1 cells were grown in alpha MEM medium containing 10% dialyzed fetal calf serum, on a gyrorotatory shaker at 37°C. The average doubling time was 12 hours and growth rate was exponential up to 0.8-1.0 x 10^6 cells/ml. The cells were routinely tested for mycoplasma and found to be negative.

Determination of Deoxyribonucleoside Triphosphates

The method finally developed was as follows.

Extraction and neutralization. Between 0.25 x 10^7 and 4.0 x 10^7 cells were centrifuged at 1000 g for 2 min at 4°C. The medium was aspirated and the pellet was extracted with 0.4 M PCA for 20 min on ice. The PCA contained [^{3}H]adenosine for determination of dilution. After centrifugation the supernatant was neutralized by extraction with a 0.5 M solution of Alamine 336 (tricapryl tertiary amine) in Freon-TF (trichlorotrifluoroethane)

(6). Neutralized extracts were stored at -20°C. Analysis of nucleotides in samples stored for several weeks showed no evidence of breakdown.

Assay Procedures. The following components were common to both the dATP and dTTP assay, in a final volume of 180 µl: 0.02 A_{260} units poly[d(AT)], 1.8 µmoles $MgCl_2$, 1.8 µmoles dAMP, 18 µmoles Hepes buffer, pH 7.4, and 0.75 Richardson units of DNA polymerase I (7). As well, the dATP assay contained 100 pmoles (0.5 µCi) [^3H]dTTP, and 0 to 75 pmoles dATP standard. The dTTP assay contained 100 pmoles (0.5 µCi) [^3H]dATP and 0 to 75 pmoles dTTP standards.

The following components were common to both the dGTP and the dCTP assays, in a final volume of 180 µl: 0.02 A_{260} units poly[d(IC)], 1.8 µmoles $MgCl_2$, 1.8 µmoles dAMP, and 18 µmoles Hepes buffer, pH 7.4. As well, the dGTP assay contained 100 pmoles (2.2 µCi) [^3H]dCTP, 0 to 10 pmoles dGTP standards and 1.9 units DNA polymerase I. The dCTP assay contained 240 pmoles (0.5 µCi) [^3H]dGTP, 0 to 200 pmoles dCTP standard and 3.0 units DNA polymerase I.

The reaction was started by the addition of the enzyme, followed by incubation at 37°C. At each of several time points, aliquots were removed and spotted on squares of Whatman 3MM filter paper which had been wetted with 200 µl of 2% sodium pyrophosphate. The squares were washed (3 x 15 min) with a solution of 5% TCA and 1% sodium pyrophosphate (20 ml/square), then rinsed once with 95% ethanol and finally washed (1 x 15 min) with 95% ethanol. The radioactivity on the dried filters was determined using a toluene-based scintillation fluid.

Calculations. The deoxyribonucleoside triphosphates in the cell extracts being assayed will dilute the specific activities of the radioactive nucleotides used in the assay. This will have two effects, (a) the background radioactivity (due to terminal addition of single deoxyribonucleotides, see below), and (b) the assay values themselves, will both be lowered in proportion to the amount of isotope dilution that has occurred.

The following derivation provides an equation that can be used to correct both background and assay values for the isotope dilution caused by the sample. The symbols used are defined as follows:

dNTP1 and dNTP2:	deoxyribonucleoside triphosphate 1 and 2 which are incoporated into the alternating copolymer.
P1:	pmoles of radioactive dNTP2 per tube in the dNTP1 assay.
P2:	pmoles of radioactive dNTP1 per tube in the dNTP2 assay.
d1 and d2:	dilution of the radioactive nucleotide caused by the sample in the dNTP1 and dNTP2 assays, respectively.
C1 and C2:	Actual radioactivity incorporated into the polymer in the dNTP1 and

dNTP2 assays, respectively.

Y1 and Y2: Dilution-corrected radioactivity incorporated into the polymer

m1 and m2: slope of the standard curve in each assay.

X1 and X2: pmoles of dNTP1 and dNTP2 respectively in the sample, or standard, per tube.

b1 and b2: Background incorporation in the assays, i.e. the Y-intercepts of the standard curves.

V1 and V2: Volumes of sample used in each of the assays.

Since the standard curves are linear the following relationships can be defined:

1) $Y1 = m1X1 + b1$

2) $Y2 = m2X2 + b2$

But the dilution-corrected cpm (Y) is equal to the actual radioactivity incorporated (C) divided by the isotopic-dilution caused by the sample (d).

3) $Y1 = \frac{C1}{d1}$

4) $Y2 = \frac{C2}{d2}$

The isotopic-dilution, when corrected for the volume of sample used in each tube, can be expressed as:

5) $d1 = \dfrac{P1}{P1 + \dfrac{X2V1}{V2}} = \dfrac{P1V2}{P1V2 + X2V1}$

6) $$d2 = \frac{P2}{P2 + \frac{X1V2}{V1}} = \frac{P2V1}{P2V1 + X1V2}$$

These equations can be solved for X1 and X2:

$$X1 = \frac{(C1P1P2m2V2 + C1C2P2V1 - P2C1b2V1 - b1P1P2m2V2)}{V2(P1P2m1m2 - C1C2)}$$

$$X2 = \frac{(C2P1P2m1V1 + C1C2P1V2 - P1C2b1V2 - b2P1P2m1V1)}{V1(P1P2m1m2 - C1C2)}$$

We have incorporated these equations into a computer program, written in APL, which also includes linear regression analysis. The program determines the slopes and Y-intercepts of the standard curves, and then uses this information in the solution of the equations for X1 and X2.

Controls. Background incorporation (i.e., in the absence of the limiting unlabeled deoxyribonucleotide) was always measured. In addition, standards were added to cell extracts to determine if the assay was affected by the extracts, and checks were made to demonstrate that the assays were independent of the amount of extract used. Finally, time courses were always performed both with standards and with each cell extract to ensure that the maximum incorporation was reached at the same time under all conditions.

RESULTS

Purity of Reagents. The purity of the unlabeled deoxyribonucleoside triphosphates was 90-98% (molar %) as determined by HPLC. The impurities were deoxyribonucleoside monodiphosphates, which have the same extinction coefficients as the triphosphates. The concentration of the standard solutions were determined by measuring the u.v. absorbance and correcting for the presence of mono- and diphosphates. The standard nucleotide solutions were stable for several months at -20°C.

The radioactive deoxyribonucleoside triphosphates were supplied and stored in 50% ethanol. Ethanol and tritiated water were removed by lyophilization followed by dissolution in 100 mM Hepes, pH 7.4. These solutions were stable for several months at -20°C.

DNA polymerase I was supplied and stored in 50% glycerol, pH 7.0. A working solution was prepared by diluting the stock solution with 50 mM Tris-HCl, pH 7.8, containing 12 mg/ml bovine serum albumin. This solution was stored not longer than one month. Storage in Hepes buffer, pH 7.4, resulted in a greater rate of loss of activity than storage in Tris buffer at pH 7.8.

The lots of DNA polymerase I from *E. coli*, that have been purchased from Boehringer Mannheim Corp., to date have not been contaminated with phosphatase activity; however, samples obtained from other sources have been. Each new batch of enzyme was checked for phosphatase activity by incubating all the assay components, except the template-primer and the limiting, unlabelled deoxyribonucleotide. The formation of radioactive mono- and diphosphates indicates phosphatase activity. (The formation of monophosphates only might indicate either phosphatase activity or contaminating DNA which would serve as a primer-template and lead to the formation of monophosphates as a result of polymerization and subsequent 3'→5' exonuclease activity.)

DNA polymerase I itself does not catalyze the formation of monophosphates from triphosphates, via 3'→5' exonuclease, until virtually all the limiting triphosphate has been incorporated into the primer-template (8). To check for 3'→5' exonuclease activity, the DNA polymerase I was incubated in the presence of all the assay components, but with limiting radioactive triphosphate and excess unlabeled triphosphate. The formation of labeled monophosphate did not occur until virtually all the labeled triphosphate had been incorporated into the polymer.

Optimum concentrations. The optimum Mg^{+2} concentration for the polymerization reaction using poly[d(AT)] or poly[d(IC)] was between 10 and 20 mM (Fig. 1); 10 mM $MgCl_2$ was used in subsequent assays.

Poly[d(IC)] was used in the dGTP/dCTP assays instead of poly[d(GC)] because at equal concentrations the rate of polymerization with poly[d(IC)] was 3.6 times the rate with poly[d(GC)]. Poly[d(IC)] at 0.02, 0.04 and 0.08 A_{260} units/tube gave equal rates of polymerization in the dGTP and dCTP assays, and poly[d(AT)] at 0.02, 0.04 and 0.1 A_{260} units/tube gave equal rates of reaction in the dATP/dTTP assays.

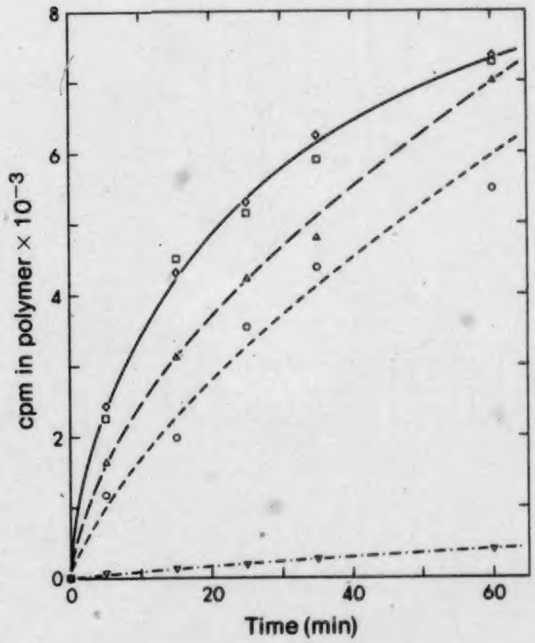

FIG. 1. Effect of Mg^{2+} concentration on the rate of polymerization of $[^3H]$dGTP and dCTP using poly[d(IC)]. Concentrations of $MgCl_2$ were 0 (▽), 5 mM (△), 10 mM (□), 20 mM (◇), and 40 mM (○).

Background radioactivity. Background incorporation (i.e., in the absence of the limiting unlabelled deoxyribonucleotide) increases with the amount of primer-template present (Fig. 2 and 3) and with the specific activity of the labelled deoxyribonucleotide (Fig. 4).

According to Baumunk and Friedman (9), background incorporation is the result of terminal addition of a single deoxyribonucleotide. Terminal addition of the labelled deoxyribonucleotide in the absence of the other complementary nucleotides can be accounted for by three explanations: 1) the DNA polymerase preparation contains terminal transferase activity; 2) the DNA polymerase has lost its ability to carry out faithful copying of the template; 3) the DNA polymerase performs a limited repair synthesis at those ends of the primer-template that are able to accept the labelled deoxyribonucleotide.

Terminal transferase does not require a template and therefore any deoxyribonucleoside triphosphate would compete with the labelled deoxyribonucleotide for incorporation. Similarly, the loss of the ability to faithfully copy a template would produce the same result. However, with this enzyme preparation the incorporation of [^{3}H]dTTP into poly[d(AT)], in the presence and absence of dATP, was unaffected by dCTP or dGTP at the same concentration as the [^{3}H]dTTP, but did decrease in proportion to the amount of unlabeled dTTP added. As well, the incorporation of [^{3}H]dCTP into poly[d(IC)] in the presence and absence of

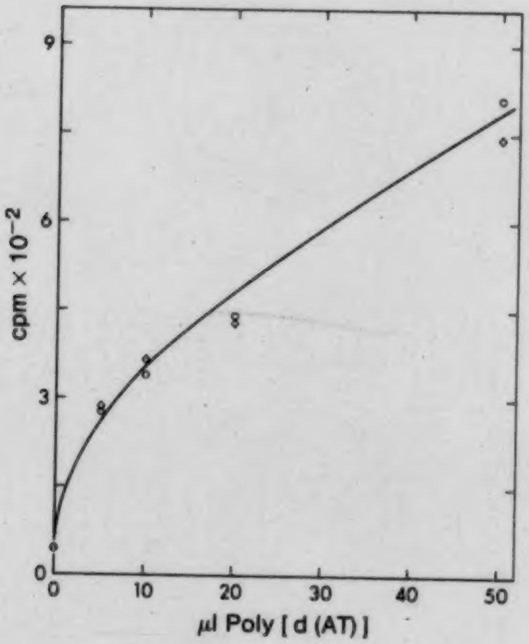

FIG. 2. Effect of poly[d(AT)] concentration on background incorporation. Incubation time: 60 min (◇), 90 min (○). The concentration of stock poly[d(AT)] was 0.002 A_{260} units per µl.

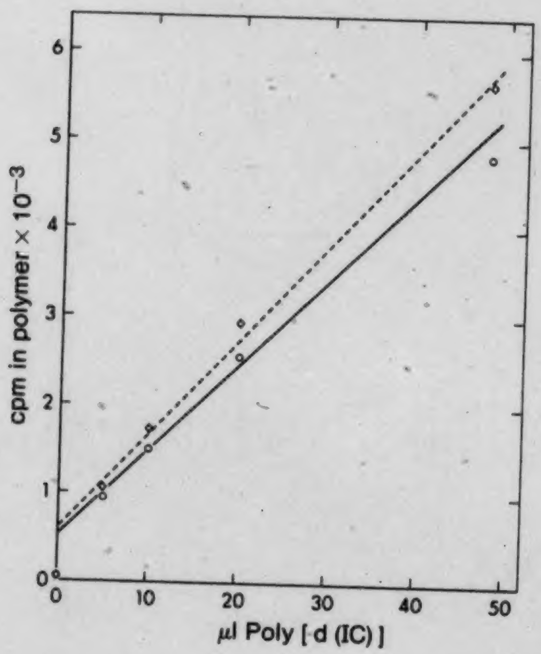

FIG. 3. Effect of poly[d(IC)] concentration on background incorporation. Incubation time: 40 min (◇), 60 min (○). The concentration of stock poly[d(AT)] was 0.002 A_{260} units per µl.

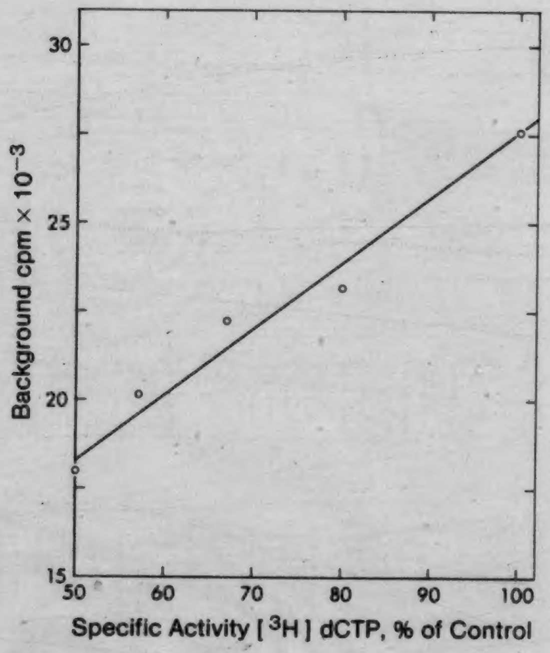

FIG. 4. Effect of dilution of specific activity on background incorporation.

dGTP was unaffected by addition of dATP or dTTP at the same concentration as the [^{3}H]dCTP, but did decrease in proportion to the amount of unlabelled dCTP added.

Explanation 3) is consistent with the above data and also with the fact that the background incorporation increases with increasing amounts of primer-template and with increasing specific activity of the labelled nucleotide. (The background incorporation is also sensitive to 3'-5' exonuclease activity, as the control time course in Fig. 5 shows.)

Incubation Time. In order for the results to be independant of the rate of polymerization and subsequent exonucleation the maximum incorporation of labelled deoxyribonucleotide into the primer-template must be measured. The time for achieving maximum incorporation varied among enzyme lots. The time of maximum incorporation was determined each time new solutions were prepared and also when extracts from cells tested with a new drug were assayed, in case the drug or its metabolites might inhibit the assay. Samples at three times were taken during each routine assay to ensure that the maximum incorporation had been reached.

Exonuclease Inhibitor. In order to extend the ideal sampling period, 2'-deoxy-5'-AMP was used to inhibit 3'-5' exonuclease activity. Byrnes et al. (10) have reported that 1 mM dAMP inhibits the 3'-5' exonuclease activity of DNA polymerase I by 80%. We have found with poly[d(AT)] as the substrate, that 2.5 mM dAMP inhibits the exonuclease

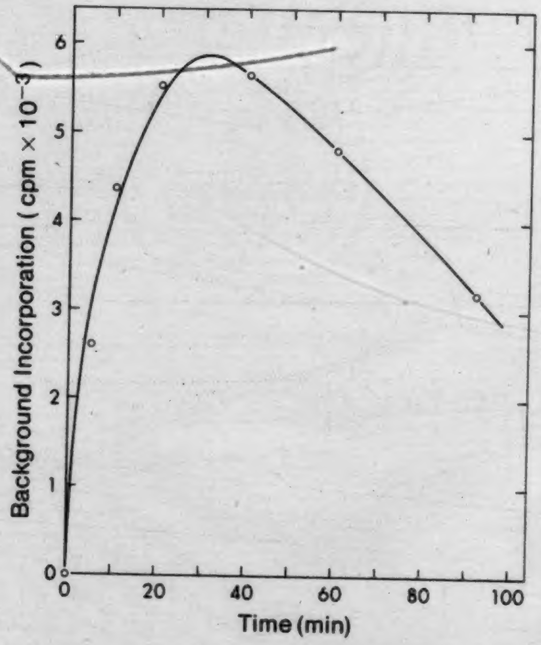

FIG. 5. Exonuclease activity. Time course of background incorporation of [^{3}H]dCTP into poly[d(IC)] in the absence of an exonuclease inhibitor.

activity by 71% while not affecting the polymerase activity (Fig. 6). With poly[d(IC)] as the substrate, 2.5 mM dAMP inhibits the 3'→5' exonuclease activity by more than 90%. Maximum inhibition for the exonuclease with either substrate was achieved with 10 mM dAMP. This concentration was used routinely in the assay.

Incubation Temperature. According to Kornberg (8), an increase in temperature from 30°C to 37°C enhances the hydrolysis of double-stranded DNA by *E. coli* 3'→5' exonuclease by a factor of 10. Studies with synthetic DNA polymers have confirmed that the 3'→5' exonuclease requires a frayed or unpaired 3'-hydroxyl terminus as a substrate. Prior to the use of dAMP to inhibit the 3'→5' exonuclease the effect of a decrease in temperature on the rate of exonucleation was determined. A decrease in temperature from 37°C to 25°C reduced the rate of 3'→5' exonucleation by approximately 55%, and reduced the rate of polymerization by 35%. This small differential effect of temperature on exonucleation verses polymerization was not very useful, especially when compared to the effect of dAMP on exonucleation. Therefore, incubation was routinely carried out at 37°C.

Isolation of product. Several methods of separating the radioactive precursor from the radioactive product have been tried. Table 1 compares the results obtained with precipitation and filtration onto glass, nitrocellulose, Millipore (cellulose acetate, 0.45 μCi) and Whatman

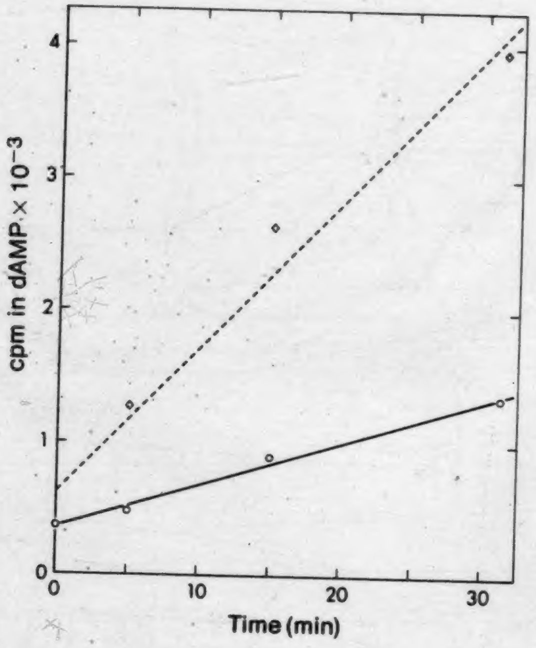

FIG. 6. Formation of radioactive dAMP from radioactive poly[d(AT)] in the presence (○) and absence (◇) of dAMP.

Table 1

Comparison of Methods for Separating Precursor from Product.

Filter Type	% Recovery of Polymer	% Retention of Free Nucleotides ([^{3}H]dATP)
Glass Fiber	86	3
Nitrocellulose	100	0.8
Millipore (Cellulose Acetate)	100	0.3
Whatman 3 MM	39	0.1
Standard Method	94	0.2

Samples containing either [^{3}H]poly[d(AT)] or [^{3}H]dATP were precipitated with 10% TCA, filtered and washed with 10% TCA followed by 95% ethanol. The recovery of the polymer and the retention of the free nucleotide was determined by comparing these values with those obtained by spotting the samples directly on the filter, then drying and measuring radioactivity.

3MM, filters, with the standard method finally used (applying portions of incubation mixture directly to Whatman 3MM filters which had been wetted with 2% sodium pyrophosphate).

Precipitation and filtration onto nitrocellulose or Millipore filters gave results as good or better than the standard method but was more time consuming. Glass fiber filters gave poor recovery of the polymer and substantial retention of the free nucleotide. Whatman 3MM filters gave very poor recovery of the polymer. The standard method has the advantage of speed since samples are spotted directly on the paper during the incubation, thus eliminating the precipitation and filtration steps, and as well large numbers of samples may be washed simultaneously.

The counting efficiency of the tritiated mononucleotides on Whatman 3MM paper in toluene based counting fluid is 5%, whereas the counting efficiency of the product polymer is 18%. This has the effect of lowering the washing background.

Effects of Cell Extracts. Effects of cell extracts on the assay were assessed in three ways: 1) time courses were performed to compare the time required to reach maximum incorporation in the presence of standard with the time required in the presence of cell extracts; 2) the volume of the extract assayed was varied to determine if the values obtained were proportional to the volume assayed; 3) known amounts of deoxyribonucleotides were added to portions of the cell extracts and the results were compared with the results for the extracts alone.

The results of these three tests indicate that cell extracts behave identically to standards in this assay.

Sensitivity. The sensitivity of this assay depends in part on the specific activity of the labeled deoxyribonucleotide. A measure of the sensitivity is the ratio of radioactivity incorporated to the pmoles of limiting deoxyribonucleotide. These values are given for each assay in Table 2.

The sensitivities of all but the dGTP assay, could be increased by increasing the specific activity of the labeled deoxyribonucleotide. In the case of the dGTP assay, the specific activity of [^{3}H]dCTP was the maximum that was commercially available.

Range. No attempt was made to determine the maximum range of the assay. The range over which the assay has been used in this laboratory is given in Table 3.

Accuracy and Reproducibility. The accuracy and reproducibility of each deoxyribonucleoside triphosphate assay are given in Table 4. These values were obtained during routine assays and thus represent the normal accuracy and reproducibility of this assay.

Conclusion. A reliable and relatively single enzymatic assay for deoxyribonucleoside triphosphate concentrations in cell extracts has been developed on the basis of the critical evaluation of the variables involved. Concentra-

Table 2

Sensitivity of Each Deoxyribonucleotide Assay.

Assay	$\frac{\text{Cpm Incorporated Per Tube}}{\text{Pmoles Limiting Deoxyribonucleotide}}$
dATP	1863
dTTP	1309
dCTP	552
dGTP	5040

Table 3

Range of Each Deoxyribonucleotide Assay.

Assay	pMoles Per Tube	
	Maximum Range Used	Normal Operating Range
dATP	0.5 - 100	5 - 75
dTTP	5 - 100	5 - 75
dCTP	5 - 200	25 - 150
dGTP	0.5 - 10	0.5 - 5

Table 4

Accuracy and Reproducibility of Each Deoxyribonucleotide Assay Under Routine Conditions

Assay	Accuracy (percent)	Reproducibility (Standard Deviation, percent)	Number of determinations
dATP	98	2.5	4
dTTP	96	5.8	4
dCTP	93	4.3	4
dGTP	78	8.5	4

tions of these nucleotides in extracts of log phase cells of our strain of Chinese hamster ovary cells (in pmole/10^6 cells) are dTTP, 58; dCTP, 223; dATP, 39; and dGTP, 12.

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CHAPTER 4

QUANTITATIVE ANALYSIS OF PURINE METABOLISM IN CHINESE HAMSTER OVARY CELLS

INTRODUCTION

Chinese hamster ovary (CHO) cells are in common use for a wide variety of biological, biochemical and pharmacological studies, some of which are related, directly or indirectly, to the purine metabolism of these cells. Although a certain amount of information regarding the purine metabolism of intact CHO cells has been acquired, particularly through the isolation and characterization of purine auxotrophs (1,2,3), this remains incomplete and depends in large part on data obtained under a wide variety of conditions. It is the purpose of this paper to report a detailed, quantitative study of purine metabolism in logarithmically growing CHO cells, including analysis of purine ribonucleoside di- and triphosphate and of deoxyribonucleoside triphosphate concentrations, and of pathways and rates of metabolism of radioactive adenine, hypoxanthine, guanine, guanosine, deoxyadenosine and deoxyguanosine.

MATERIALS AND METHODS

[2-^{3}H]Adenine, 12 Ci/mmol, and [2-^{3}H]hypoxanthine, 15 Ci/mmol, were obtained from Moravek Biochemicals; [2-^{3}H]-adenosine, 24 Ci/mmol, from Amersham-Searle; [8-^{3}H]guanine, 15 Ci/mmol, from New England Nuclear Corp.; [8-^{3}H]guanosine, 5.7 Ci/mmol, and [8-^{3}H]deoxyguanosine, 9.0 Ci/mmol, from Schwarz-Mann Co.; and [8-^{3}H]deoxyadenosine 11 Ci/mmol, from ICN Pharmaceuticals Inc.; [^{3}H]dATP, 11 Ci/mmol, [^{3}H]-dTTP, 14 Ci/mmol, [^{3}H]dCTP, 16.8 Ci/mmol, and [^{3}H]dGTP, 15 Ci/mmol, were obtained from ICN Pharmaceuticals Inc.

DNA polymerase I from *E. coli* was obtained from Boehringer-Mannheim Corp., DNase I, and snake venom phosphodiesterase and alkaline phosphatase, from Sigma Chemical Co. Poly[d(IC)] was purchased from Miles Laboratories, and poly[d(AT)], non-radioactive purine and pyrimidine bases, nucleosides and nucleotides, from Sigma Chemical Co.

Cell Culture. Chinese hamster ovary-K1 cells, obtained from Dr. G. Whitmore (Ontario Cancer Institute, Toronto, Ontario), were grown in alpha-MEM medium containing 10% dialyzed fetal calf serum (Grand Island Biological Co.). The cells were grown in 125 ml bottles on a Model G-2 Gyrotary shaker (New Brunswick Scientific Co.) at 200 rpm. The average doubling time was 12 h and the growth rate was exponential to between 0.8 and 1.0 x 10^6 cells/ml. The cells were routinely tested for mycoplasma by the Department of Medical Bacteriology, University of Alberta, Edmonton, Alberta and found to be negative. Cell density was determined using a Model Z_F Coulter counter.

Cell Extraction. Preparation of extracts for nucleotide pool size measurements was as follows: 0.25 to 4.0 x 10^7 cells were centrifuged at 1000 g for 2 min at 4°C. The medium was aspirated and the tube recentrifuged at 1000 g for 5 sec to remove medium from the centrifuge tube wall. The pellet was extracted on ice with 0.4 M PCA containing [^{3}H]adenosine for determination of dilution. After 30 min the extract was centrifuged and the supernatant was removed and neutralized by extraction with 0.5 M Alamine 336 (tricapryl tertiary amine) in Freon-TF (trichlorotrifluoroethane) (4). Supernatants were stored at -20°C. HPLC analysis of nucleotides in samples stored for several weeks showed no nucleotide breakdown.

Ribonucleotide Concentrations. Ribonucleotide concentrations were measured by HPLC using a modified Varian Aerograph 1000 liquid chromatograph equipped with a Spectra-Physics Model 740P pump, a Waters Associates U6K injector, and a Spectra-Physics Autolab Minigrator. A Partisil 10 SAX anion exchange column (Whatman) was used, and the nucleotides were routinely eluted isocratically at 38°C with 0.25 M KH_2PO_4, 0.5 M KCl, pH 4.5 at 1.3 ml/min; this allowed quantitation of ADP and the triphosphates. When values for the mono-, di- and triphosphates were required, a linear gradient from 0.015 M KH_2PO_4, pH 4.0 to 0.5 M KH_2PO_4, 1.0 M KCl, pH 4.5 was used. Detection was at 254 nm and at 0.02 absorbance units full scale. The peaks were automatically integrated and also checked by planimetry. The absolute amounts of nucleotides in the sample were calculated on the basis of peak areas of nucleotide standards which were chromatographed frequently.

Deoxyribonucleoside Triphosphate Concentrations. The following components were common to both the dATP and dTTP assay, in a final volume of 180 µl: 0.02 A_{260} units poly-[d(AT)], 1.8 µmoles $MgCl_2$, 1.8 µmoles dAMP, 18 µmoles Hepes buffer, pH 7.4, and 0.75 Richardson units of DNA polymerase I (5). As well, the dATP assay contained 100 pmoles (0.5 µCi) [^{3}H]dTTP, and 0 to 75 pmoles dATP standard, while the dTTP assay contained 100 pmoles (0.5 µCi) [^{3}H]dATP and 0 to 75 pmoles dTTP standard.

The following components were common to both the dGTP and the dCTP assays, in a final volume of 180 µl: 0.02 A_{260} units poly[d(IC)], 1.8 µmoles $MgCl_2$, 1.8 µmoles dAMP, and 18 µmoles Hepes buffer, pH 7.4. As well, the dGTP assay contained 100 pmoles (2.2 µCi) [^{3}H]dCTP, 0 to 10 pmoles dGTP standard and 1.9 units DNA polymerase I. The dCTP assay contained 240 pmoles (0.5 µCi) [^{3}H]dGTP, 0 to 200 pmoles dCTP standard and 3.0 units DNA polymerase I.

The reaction was started by the addition of the DNA polymerase I, followed by incubation at 37°C. At each of several time points aliquots were removed and spotted on squares of Whatman 3MM filter paper which had been wetted with 200 µl of 2% sodium pyrophosphate. The squares were washed (3 x 15 min) with a solution of 5% TCA and 1% sodium pyrophosphate (20 ml/square), then rinsed once with 95% ethanol and finally washed (1 x 15 min) with 95% ethanol. The dried filters were counted in toluene scintillation fluid containing 4 g PPO and 0.1 g POPOP per liter toluene.

Results were corrected for the washing background and for the effects of the dilution of the specific activity of the labelled deoxyribonucleotide by the sample.

The purity of the unlabelled deoxyribonucleoside triphosphates was 90-98 molar % as determined by HPLC. The impurities were deoxyribonucleoside mono- and diphosphates, which have the same extinction coefficients as the triphosphates. The concentration of the standard solutions was determined by measuring the u.v. absorbance and correcting for the presence of mono- and diphosphates. The standard nucleotide solutions were stable for several months at -20°C.

The radioactive deoxyribonucleoside triphosphates were supplied and stored in 50% ethanol. Ethanol and tritiated water were removed by lyophilization followed by dissolution in 100 mM Hepes, pH 7.4. The radiochemical purity was 86-94%. These solutions were stable for several months at -20°C.

DNA polymerase I was supplied and stored in 50% glycerol, pH 7.0. A working solution was prepared by diluting the stock solution with 50 mM Tris-HCl, pH 7.8 containing 12 mg/ml bovine serum albumin. This solution was stored not longer than 1 month.

The lots of E. coli DNA polymerase I purchased from Boehringer Mannheim Corp., have not been contaminated with phosphatase activity, but samples obtained from other sources have been. Each new batch of enzyme was checked for phosphatase activity.

The following controls were performed: background incorporation (i.e., in the absence of the limiting nonradioactive deoxyribonucleotide) was always measured. In addition, standards were added to cell extracts to determine if the assay was affected by the extracts, and checks were made to demonstrate that the assays were independent of the amount of extract used. Finally, time courses were always performed both with standards and with each cell extract to ensure that the maximum incorporation was reached at the same time under all conditions.

Cell Incubations and Sample Preparation. CHO-K1 cells at a density of 200,000 to 400,000 cells/ml were labelled with radioactive precursors at concentrations between 0.20 and 1.8 μM and at specific activities between 5.5 and 50 Ci/mmole. Portions of 1 or 5 ml were removed over a 90 min time course. The cells were pelleted by centrifugation and extracted with 50 μl of ice-cold 0.4 M PCA containing [^{14}C]-adenine for determination of dilution. After 30 min on ice, the samples were centrifuged. The supernatant was removed and neutralized with an equal volume of 0.5 M Alamine-336 in Freon-TF (4). 10 μl were counted in Triton X-100 counting fluid (0.4% PPO, 0.02% POPOP, in 1 part Triton

X-100 and 2 parts toluene) to measure the [^{14}C]adenine radioactivity which was used to calculate the dilution occurring during extraction. The tritium in the extract represented mainly label incorporated into nucleotides as well as some label from the original precursor. The neutralized extracts were stored at -20°C.

The acid-insoluble pellet, containing labelled DNA and RNA, was washed 3 times with ice-cold 0.4 M PCA, then dissolved in 200 μl 0.2 N KOH and incubated at 37°C for 18 h to hydrolyze the RNA. The DNA was precipitated on ice by addition of 4 M PCA to a final concentration of 0.3 M. After centrifugation, the supernatant containing the 2'- and 3'-monophosphates from the hydrolyzed RNA, was removed and neutralized with an equal volume of Alamine-Freon. 25 μl were counted in Triton-X100 counting fluid before chromatography to determine the total radioactivity in the RNA nucleotides and the remainder was stored at -20°C. The DNA pellet was washed 3 times with ice-cold 0.4 M PCA, then dissolved in 50 μl of 100 mM Tris-buffer, pH 8.0. $MgCl_2$, NaCl, and DNase I were added to final concentrations of 2.5 mM, 5.0 mM and 50 μg/ml respectively, and the solution was incubated ca. 18 h at 37°C. Ammonium acetate, $MgCl_2$, snake venom phosphodiesterase, and alkaline phosphatase were added to final concentrations of 100 mM, 2 mM, 50 μg/ml (10 x 10^{-3} U), and 165 μg/ml (182 U), respectively, and the solution was incubated at 37°C for ca. 18 h. If alkaline phosphatase was not used, conditions which resulted in complete

DNA hydrolysis produced both the deoxyribonucleoside-monophosphates and deoxyribonucleosides, complicating the separation procedure. 25 μl of the DNA hydrolysate were counted in Triton X-100 counting fluid to determine the total radioactivity before chromatography. The DNA hydrolysate was stored at -20°C.

Chromatography. The acid soluble purine and pyrimidine ribo- and deoxyribonucleotides were separated by two dimensional chromatography on PEI-cellulose thin layer plates using a modification of the method of Crabtree and Henderson (6). The plates were prepared by attaching a wick and washing overnight with 1.8% ammonium formate, 2% boric acid, pH 7.0, followed by an overnight wash with 50% methanol in water. After drying, the plates were stored at 4°C over a desiccant. Markers and 10 or 20 μl of the extract were spotted and the plates were run overnight in 50% methanol in water to wash bases and nucleosides onto the wick. The wicks were removed, the plates dried, and fresh wicks attached. The plates were developed to 8 cm above the origin with 1.8 M ammonium formate, 2% boric acid, pH 7.0 (immediately) followed by development in 3.3 M ammonium formate, 4.2% boric acid, pH 7.0 until the leading marker was near the wick. The wick was removed and the plates were immersed in methanol for 15 min. After drying, the plates were developed in the second dimension to 2.5 cm above the origin with 0.5 M sodium formate pH 3.4, then to 8 cm above the origin in 2.0 M sodium formate pH 3.4, and finally up to the wick or until the triphosphates were well separated, in 4.0 M sodium formate, pH 3.4. At times, the leading monophosphates were run onto the wicks in order to achieve a better separation. After drying and removal of the wicks, the plates were dipped in methanol to remove the salt in preparation for oxidation. The spots were visualized with u.v. light and scraped onto Whatman No. 1 paper and oxidized, in a Packard 305 oxidizer, to $[^3H]H_2O$. The counting efficiency was 30-34% and the recovery at the oxidation step ranged from 90 to 95%. The effects of carry-over of $[^3H]H_2O$ from one sample to another were minimized both by adjusting the order in which the samples were burned and by the frequent oxidation of blanks.

The 2'- and 3'-monophosphates from hydrolyzed RNA were separated on PEI-cellulose plates using a one-dimensional system. The plates were prepared by attaching a wick and washing overnight with 2.0 M sodium formate, pH 3.4, followed by an overnight wash with 50% methanol in water. Purine or pyrimidine 2'- and 3'-monophosphate markers plus 10 to 50 µl of the sample were spotted over a 2 cm width to prevent streaking during development caused by salts in the sample. The plates were developed to the top in 0.3 M sodium formate, pH 3.4. After drying, the plates were dipped in methanol to remove salts, the spots were visualized, scraped and oxidized. The origins were routinely scraped and oxidized to ensure that complete hydrolysis of the RNA occurred and that there was no DNA contamination in the

RNA fraction. During the development of the RNA hydrolysis procedure, the entire plate was scraped to check that no radioactivity was present in compounds other than 2'-, 3'-nucleoside monophosphates.

Markers plus 25 to 100 µl of the DNA hydrolysate were streaked on cellulose thin-layer plates. The samples containing labelled purine deoxyribonucleosides were developed in isobutyric acid: ammonium hydroxide: 5% EDTA in water (200:9:14). The samples containing labelled pyrimidine deoxyribonucleosides were developed in ethyl acetate: isopropanol: water (50:18:10). After development, the plates were dried, the spots were visualized and scraped. The spots were either oxidized or scraped into scintillation vials and eluted with 1 ml of water for 2 days and then counted in Triton X-100 counting fluid. When the spots were oxidized, the amount of label in acid soluble pools, RNA and DNA could be compared directly; however, when the DNA nucleosides were eluted, the counts had to be corrected for both elution and counting efficiency. To do this, standard radioactive solutions of each deoxyribonucleoside were run with each batch of chromatograms, with one set eluted and the other oxidized. The origins were routinely counted to check for incomplete DNA hydrolysis and when the procedure was being developed, the entire plate was oxidized to ensure that the label was present only in deoxyribonucleosides.

The entire RNA and DNA isolation and hydrolysis procedure was evaluated using the acid insoluble pellet of cells incubated with [^{3}H]TdR. No significant amount of label from thymidine DNA was found in the RNA fraction, indicating both that little cross contamination was occurring and that tritium exchange with water was not occurring under the conditions of alkaline RNA hydrolysis.

RESULTS AND DISCUSSION

Two types of analyses were carried out to characterize quantitatively the purine metabolism of cultured CHO-K1 cells. First, concentrations of ribonucleoside di- and triphosphates and of deoxyribonucleoside triphosphates were determined. Second, alternative pathways of metabolism of several radioactive precursors were measured.

Nucleotide Concentrations. Concentrations of purine nucleotides in CHO cells are reported in Table 1. Ribonucleotide pools were approximately 100 times larger than the corresponding deoxyribonucleotide pools; however, they are not turning over at 0.01 times the rate of the deoxyribonucleotide pools. In fact, they are turning over substantially faster than this since these pools are not only precursors for the deoxyribonucleotides, but also for RNA, conjugated nucleotides and coenzymes such as NAD, and in addition they are consumed by reactions such as ADP ribosylation. The lack of correlation between pool size and turnover rate is further demonstrated by the fact that the dATP pool is 3-fold larger than the dGTP pool although

TABLE 1. Purine Ribo- and Deoxyribonucleotide Concentrations in CHO-K1 Cells

Nucleotide	Concentration (pmoles/10^6 cells)
ATP	5,580
ADP	378
GTP	1,070
GDP	162
dATP	39
dGTP	12

their rates of incorporation into DNA must be similar.

Radioactive Precursor Studies. CHO cells were next incubated with various radioactive precursors, samples were taken at different times up to 90 min, and the radioactivity in individual acid-soluble, RNA and DNA nucleotides was determined. For all of the precursors used the rate of labelling of DNA and of RNA became linear within 20 to 25 min, and the data presented here are from incubations (usually 30 min) when the incorporation of radioactivity into nucleic acids was linear, and hence when the system was in a steady state. Incubations were continued for longer periods to ensure that the labeling rate did not decline, due either to radiation effects or to depletion of precursor.

In most cases, only one low concentration of precursors was used, hence these results apply to tracer concentrations. The possibility that higher concentrations might have been metabolized differently from a quantitative point of view must be considered.

A major assumption of this work was that the addition of labelled precursors did not significantly perturb the metabolism of the cell. The concentration of the precursors added was very low (0.6 to 1.8 μM), and since much higher concentrations (10 to 100X) of cold precursors were required to cause measurable nucleoside triphosphate pool size changes (Henderson, J.F. and Lowe, J.K., unpublished results), it is very unlikely that these low levels contributed significantly to the total metabolic pools. In order to measure accurately the amount of radioactivity in very small pools, precursors of high specific activitiy (5.5 to 50 Ci/mmol) were used. Although long term exposures to these high specific activities would likely have been toxic (review:7) these experiments were short term and the nucleotide pool, DNA, and RNA labelling kinetics indicated. that these cells were not affected by these exposures.

Table 2 shows the results of 30 min incubations of CHO cells with tritiated adenine, hypoxanthine, guanine and guanosine, a 75 min incubation with deoxyguanosine, and a 40 min incubation with deoxyadenosine plus 2'-deoxycoformycin, an inhibitor of adenosine deaminase. The data given in Table 2 represent the raw data from which other results were calculated.

In Table 3, the total conversion to nucleotides for each precursor has been calculated as the sum of radioactivity in acid-soluble and nucleic acid nucleotides (as reported in Table 2). This in turn is divided (for each precursor) into the total radioactivity in the acid-soluble nucleotides, in RNA and in DNA. (Any radioactivity in nucleosides and bases, which would be predominantly in the medium rather than within the cells, was not measured.)

Table 3 also lists the specific activity of each of the precursors used in these experiments. By comparing these values with the values for the total conversion of radioactivity to nucleotides it is possible to estimate the

TABLE 2. Incorporation of Radioactive Precursors into Purine Nucleotides

Nucleotide	Incorporation (counts/min per 10^6 cells)					
	Precursors					
	Adenine	Hypoxanthine	Guanine	Guanosine	Deoxyguanosine	Deoxyadenosine
ATP	3,500,000	709,000	11,200	21,100	21,700	21,000
ADP	219,000	113,000	1,570	3,430	4,050	3,970
AMP	31,000	17,700	361	1,920	1,670	1,110
IMP	trace	5,220	403	trace	1,010	trace
GTP	24,400	151,000	152,000	1,060,000	1,180,000	trace
GDP	747	27,400	20,400	63,300	138,000	trace
GMP	815	3,310	2,740	4,740	31,900	trace
dATP	9,370	2,450	264	trace	7,690	20,800
dADP	866	591	472	trace	3,830	2,730
dAMP	1,410	trace	42	trace	662	1,430
dGTP	trace	4,400	1,830	5,770	60,100	trace
dGDP	trace	802	292	trace	8,480	trace
dGMP	trace	1,270	4,020	trace	2,370	trace

(... cont'd. of Table 2 ...)

Nucleotide	Incorporation (counts/min per 10^6 cells)					
	Precursors					
	Adenine	Hypoxanthine	Guanine	Guanosine	Deoxyguanosine	Deoxyadenosine
RNA-A	33,500	39,600	3,230	5,670	11,900	9,760
RNA-G	2,520	57,400	64,400	136,000	3,000,000	9,760
DNA-A	8,580	9,240	1,050	1,240	10,700	43,500
DNA-G	trace	7,850	6,510	29,700	233,000	332

TABLE 3. Metabolism of Radioactive Purines in CHO-K1 Cells

Precursor	Specific activity	Sampling time	Total conversion to nucleotides	Amount in acid-soluble nucleotides	Amount in RNA	Amount in DNA
	(Ci/mmol)	(min)	(counts/min per 10^6 cells)			
Adenine	12	30	3,830,000	3,780,000	36,000	8,580
Hypoxanthine	15	30	1,150,000	1,030,000	97,100	17,100
Guanine	5.5	30	271,000	196,000	67,700	7,550
Guanosine	5.5	30	1,330,000	1,160,000	142,000	30,900
Deoxyguanosine	9	75	4,730,000	1,470,000	3,020,000	244,000
Deoxyadenosine	11	40	105,000	51,000	9,760	43,900

relative efficiencies of utilization of these precursors. For example, although the specific activity of hypoxanthine was 1.3-fold higher than that of adenine, the total conversion of radioactivity to nucleotides from adenine was 3.2-fold higher than for hypoxanthine, demonstrating that adenine was taken up more readily than hypoxanthine. The concentrations of adenine and hypoxanthine were 0.8 and 0.7 μM respectively, so the difference in uptake was not a result of a concentration difference, but reflects a difference in either the K_m or the V_{max} of the rate limiting step for precursor utilization. Deoxyadenosine was taken up the least efficiently of any precursor. It was used at approximately the same specific activity and concentration as adenine, and yet the total conversion to nucleotides of adenine was 38-fold greater than that of deoxyadenosine, even though the incubation time with deoxyadenosine was 10 min longer than with adenine.

Guanine was taken up less efficiently than guanosine, and the total conversion to nucleotides from guanosine was 5-fold higher, although the precursor concentrations were the same. The basis of this difference is not known.

Figs. 1 to 6 express the amount of radioactivity that had flowed through a given reaction as a percentage of the total amount of radioactivity that was converted to nucleotides.

This way of expressing the data will be illustrated by considering the metabolism of ADP in Fig. 1. Thus when

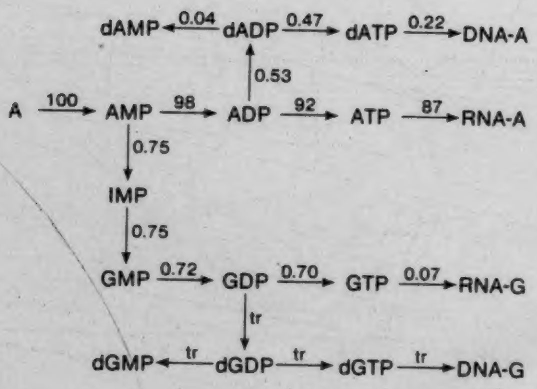

FIG. 1. Metabolism of radioactive adenine. The amount of radioactivity from the precursor that has flowed through a given reaction (expressed as a percentage of the total amount of radioactivity converted to nucleotides) is given on the arrows representing individual reactions.

radioactive adenine was the precursor, the total incorporation of radioactivity into nucleotides was 3,830,000 counts/min per 10^6 cells. Of these, 3,750,000 counts/min per 10^6 cells, or 98%, flowed into the ADP pool, while 3,540,000 counts/min per 10^6 cells, or 92.5%, flowed out, leaving 5.5% of the radioactivity in the ADP pool. Of the radioactivity which flowed through the ADP pool, 3,520,000 counts/min per 10^6 cells, or 92%, were phosphorylated, while 19,200 counts/min per 10^6 cells, or 0.53% were reduced by ribonucleotide reductase. Thus, the ratio of reduction of ADP to phosphorylation was 0.53 to 92 or 1 to 174.

In the analysis of the results, particular attention will be paid to the partition of the radioactive precursor flow at branch points in the pathway, as illustrated in the example above. Since DNA and RNA are "sinks" for the radioactive label (review:7), longer incubations will increase the proportion of radioactivity present in nucleic acids without affecting the absolute amount in the acid soluble nucleotides or the relative amounts in individual nucleotides. A significant amount of turnover of radioactivity into RNA occurs; nevertheless, RNA is still a net sink for radioactivity (8).

Adenine Metabolism. Adenine is almost certainly converted to adenylate by adenine phosphoribosyltransferase rather than by adenosine phosphorylase plus adenosine kinase (9). When the incorporation of adenine into RNA and

DNA is linear, each pool in the direct pathway between adenine and RNA and DNA will have reached its maximum specific activity. Pools in sections of the pathway with only one input will contain an amount of radioactivity proportional to their size since they will all have the same specific activity. Other pools, not on the direct pathway from adenine to RNA or dAMP, will only have reached their maximum specific activities if they are turning over at approximately the same rate or faster than the ATP pool. If they are turning over much more slowly, they will require more time to reach their steady state specific activity.

Simply because a pool has reached its steady state specific activity does not necessarily mean it has the same specific activity as all the other steady state pools. For example, AMP, labeled by [^{3}H]adenine, is converted to guanine nucleotides via adenylate deaminase, inosinate dehydrogenase and guanylate synthetase. The specific acitivity of the IMP pool is diluted by de novo synthesis with the result that its steady state specific activity, and that of all the subsequent pools on the pathway, is lower than the specific activity of the adenine nucleotide pools.

The fact that most of the adenine was converted to adenine nucleotides indicates that the effective activity of adenylate deaminase, the rate limiting enzyme for guanylate synthesis from adenylate (9), was quite low.

When the system is in a steady-state it is possible to determine the relative pool sizes of compounds by comparing the amounts of radioactivity in the separate pools, as long as there is no input of unlabelled compound into the pathway at a point between the pools that are being compared. Thus, for example, the ratio of the amount of radioactivity in ATP to that in ADP should equal the ratio of their pool sizes. In this case, the ratio of radioactivity in ATP to ADP was 16 to 1 (Table 2), which compares well with the ratio of 15 to 1 found by HPLC measurements. This method can be used to determine the size of pools, such as dADP, that would be difficult to measure directly. The ratio of radioactivity in dATP to dADP was 4 to 1, and, since the dATP pool size was 39 pmoles/10^6 cells (Table 1), the calculated dADP pool size is 9.7 pmoles/10^6 cells. The relative pool sizes of ATP and dATP using this method gives a ratio of ATP to dATP of 370 to 1, while the ratio as determined by HPLC and the enzymatic assay was only 143 to 1 (Table 1). The difference in these ratios may result from the fact that these ratios were determined in separate experiments, or it may also result from dilution of the specific activity of the dATP pool by non-radioactive dATP synthesized by the salvage pathway rather than by reduction of ADP. Since the growth media contains no deoxyribonucleosides, the source of the non-radioactive dATP could be DNA repair.

The other branch point of interest is the conversion of ADP to deoxyribonucleotides; this reaction proceeded at ca. 1/180th the rate of the alternative pathway, phosphorylation to ATP.

There was more radioactivity in the dAMP pool than expected, based on the radioactivity in the dATP and dADP pools (Table 2). This may be a result of co-chromatography with some other, as yet unidentified, nucleotide.

Hypoxanthine Metabolism. Hypoxanthine is converted to inosinate by hypoxanthine-guanine phosphoribosyltransferase (Fig. 2). Approximately three-quarters of the IMP synthesized was converted to adenine nucleotides while the remainder was converted to guanine nucleotides. Since the rate of nucleic acid synthesis from adenine and guanine nucleotides is approximately the same, consumption of adenine nucleotides by other processes (e.g., ADP-ribosylation, poly A synthesis) must account for the extra adenine nucleotide usage. Hypoxanthine is an unusual precursor in that the point of entry of the label into the system, namely IMP, is the same as the entry point of unlabelled precursor from purine de novo synthesis. Thus, in the steady state situation, little dilution of the label occurs beyond the IMP pool, except possibly from catabolism of unlabelled RNA or conjugated nucleotides. Therefore, it seems a reasonable assumption that the steady-state ratio of radioactivity in ATP and GTP is equal to the ratio of their pool sizes. The ratio of radioactivity in ATP to GTP was 4.7 to

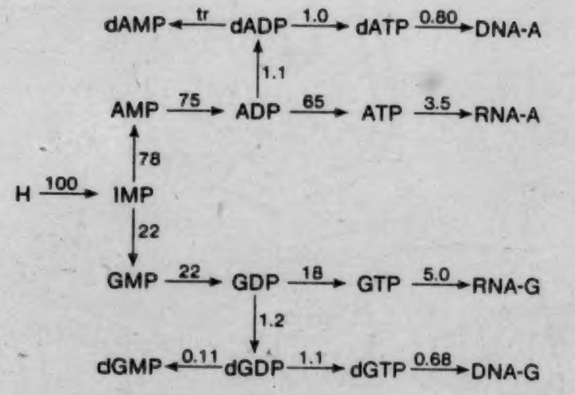

FIG. 2. Metabolism of radioactive hypoxanthine.

1 (Table 2), while the ratio of the pool sizes was 5.2 to 1 (Table 1). The size of the IMP pool, based both on the amount of radioactivity in IMP relative to ATP and on the pool size of ATP, was 42 pmoles/10^6 cells, which is approximately the same size as the dATP pool. The ratio of the amount of radioactivity in ATP to dATP was 290 to 1 which is lower than the ratio of 370 to 1 calculated when radioactive adenine was the precursor, but is still 2-fold higher than the ratio of the ATP to dATP pool sizes as determined by direct measurement (Table 1). The ratio of radioactivity in GTP to dGTP was 34 to 1, while the ratio of pool sizes, when measured directly, was 89 to 1, which is 2.6-fold higher. There was more radioactivity in the dGMP pool than one would predict from the radioactivity in the dGTP and dGDP pools (Table 2), probably due to co-chromatography with some unknown product.

Guanine Metabolism. Guanine is converted to guanylate by hypoxanthine-guanine phosphoribosyltransferase (Fig. 3). 92% of the guanylate synthesized was converted to other guanine nucleotides while the remainder was reduced by guanylate reductase, the rate limiting enzyme in the synthesis of adenine nucleotides from guanylate (10). The other branch point of interest is the conversion of GDP to deoxyribonucleotides; this reaction proceeded at ca. 1/17th the rate of the alternative pathway, phosphorylation to GTP. As was the case with hypoxanthine as the precursor, there was more radioactivity in the dGMP pool then expected.

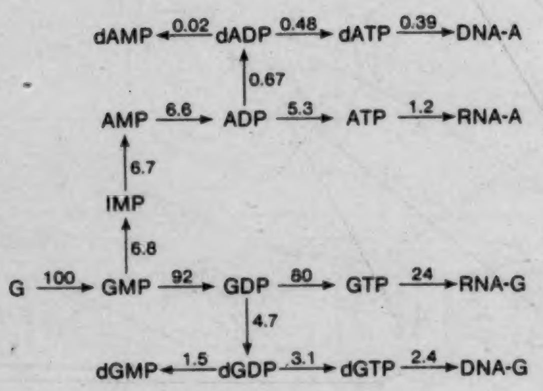

FIG. 3. Metabolism of radioactive guanine.

The ratio of radioactivity in GTP to dGTP was 83 to 1 which compares well with the ratio of the pool sizes, which was 89 to 1 (Table 1). This ratio is quite different from the one obtained when radioactive hypoxanthine was the precursor (34:1).

Guanosine Metabolism. Guanosine is converted to guanine by purine nucleoside phosphorylase (Fig. 4); very little of this enzyme activity is present in dialyzed fetal calf serum (Henderson, J.F., unpublished results). Although some conversion of guanosine directly to GMP by guanosine kinase can not be ruled out, this route is unlikely based on other studies (9). For the present purposes, the conversion of guanine to GMP has been taken as equal to the conversion of guanosine to guanine, though this point was not studied directly. Regardless of how the guanosine is converted to GMP, its routes of metabolism are virtually identical to those of guanine after that point. The data in Fig. 3 and 4 agree well, except that less guanosine than guanine was converted to adenine nucleotides and the ratio of radioactivity in RNA-guanine to DNA-guanine was higher for guanine (10:1) than for guanosine (5:1).

The ratio of radioactivity in GTP to dGTP was 109 to 1 which compares reasonably well to the value of 89 to 1 for the ratio of the pool sizes, obtained by direct measurement.

Deoxyguanosine Metabolism. Most of the deoxyguanosine was phosphorolyzed to guanine which is converted to ribonucleotides by hypoxanthine-guanine phosphoribosyltrans-

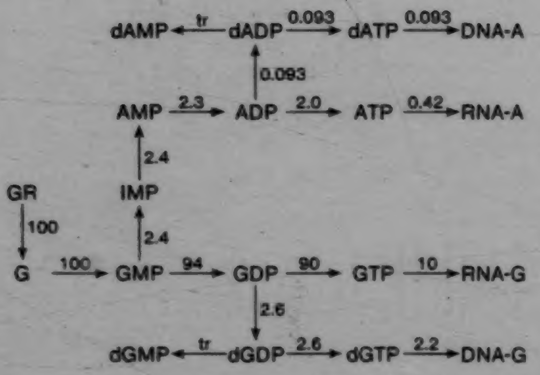

FIG. 4. Metabolism of radioactive guanosine.

ferase, hence the metabolism of deoxyguanosine was almost identical to that of guanine (Fig. 5). The phosphorolysis of deoxyguanosine is probably catalyzed by purine nucleoside phosphorylase which has little activity in dialyzed fetal calf serum. Deoxyguanosine kinase activity must be very low in these cells since at most, only 6% of the total nucleotide synthesis could be catalyzed by this enzyme. Inasmuch as radioactive deoxyguanosine nucleotides are also being synthesized by ribonucleotide reductase, the value of 6% probably is an overestimate. Based on the results of guanine and guanosine as precursors, one would expect the flow of guanine nucleotides through ribonucleotide reductase to be 3 to 5% of the total nucleotide synthesis.

However, the ratio of radioactivity in GTP to dGTP was only 20 to 1, while the ratio of the pool sizes (Table 1) was 89 to 1. Thus the specific activity of dGTP is greater than the specific activity of GTP, which indicates that not all the GdR is being phosphorolyzed to guanine.

Deoxyadenosine Metabolism. Fig. 6 shows the pathways of deoxyadenosine metabolism under conditions in which adenosine deaminase activity was completely inhibited by 2'-deoxycoformycin. Though phosphorylation to dAMP was the major primary pathway, it was striking that 34% was cleaved to adenine and converted to AMP via adenine phosphoribosyltransferase. This pathway of deoxyadenosine metabolism has been demonstrated previously in a variety of cell types (10). (It is unlikely that significant deoxyadenosine deamination is

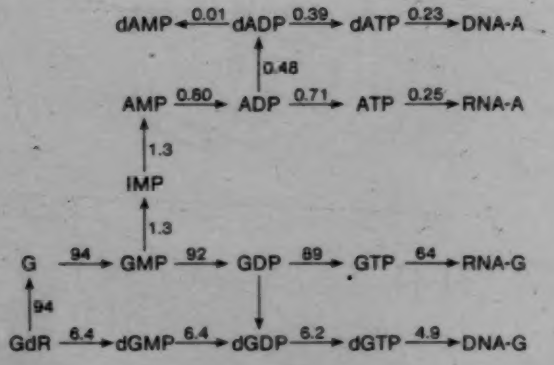

FIG. 5. Metabolism of radioactive deoxyguanosine.

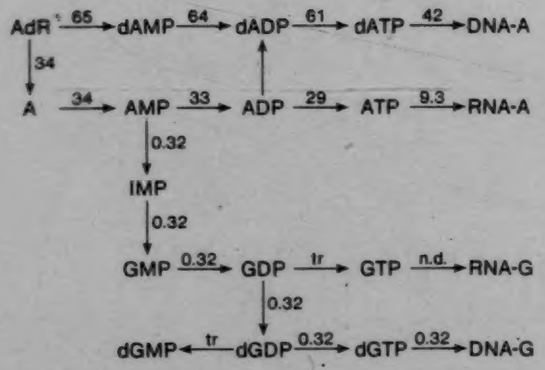

FIG. 6. Metabolism of radioactive deoxyadenosine.

occurring since the deoxyinosine produced would be phosphorolyzed to hypoxanthine which is converted to both adenine and guanine nucleotides (Fig. 2). However, under these conditions, only 0.32% of the nucleotides formed were converted to guanine nucleotides.)

In conclusion, this study has provided considerable information regarding the relative rates of alternate pathways of purine metabolism in CHO-K1 cells, and the pool sizes of some of the nucleotides which are too small to be conveniently measured by direct methods. As well, information on the relative efficiencies of utilization of the different precursors was obtained. The results of this study will be used to develop a method of measuring the true rates of DNA and RNA synthesis in control and drug treated cells, by measuring the specific activity of the precursor pools. These results may also be useful in selecting a cell line for a particular project. Eventually it may be possible to choose a cell line not only on the basis of biological characteristics such as growth rate and cloning efficiency, but also on the basis of biochemical characteristics.

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CHAPTER 5

QUANTITATIVE ANALYSIS OF PYRIMIDINE METABOLISM IN CHINESE HAMSTER OVARY CELLS

INTRODUCTION

Relatively little is known about the intermediary metabolism of pyrimidines in intact Chinese hamster ovary (CHO) cells, particularly in quantitative terms, though Patterson has reported the isolation of several CHO pyrimidine auxotrophs (1,2). It is the purpose of this paper to report a detailed, quantitative study of pyrimidine metabolism in logarithmically growing CHO cells, including analysis of pyrimidine ribonucleoside and deoxyribonucleoside triphosphate concentrations, and of pathways and rates of metabolism of radioactive uridine, cytidine, deoxycytidine, deoxyuridine and thymidine.

MATERIALS AND METHODS

[6-^{3}H]Uridine, 22.4 Ci/mmol, was obtained from New England Nuclear Corp.; [5-^{3}H]orotic acid, 19 Ci/mmol, from Schwartz-Mann Co.; [5-^{3}H]cytidine, 20 Ci/mmol, [5-^{3}H]deoxycytidine, 20 Ci/mmol, [6-^{3}H]deoxyuridine, 20 Ci/mmol, and [methyl-^{3}H]thymidine, 50 Ci/mmol, from Moravek Biochemicals.

Sources of other materials, and methods for the culture of CHO-K1 cells in alpha-MEM medium containing 10% dialyzed fetal calf serum, extraction of nucleotides, measurement of ribo- and deoxyribonucleotide concentrations, incubation with radioactive precursors, separation of individual acid-soluble ribo- and deoxyribonucleotides and nucleotides or nucleosides from RNA and DNA, and the measurement of radioactivity, are described in the preceeding paper.

RESULTS AND DISCUSSION

Pyrimidine metabolism in CHO-K1 cells was characterized quantitatively in two ways. First, concentrations of ribonucleoside and deoxyribonucleoside triphosphates were determined. In addition, the metabolism of radioactive precursors was studied to measure the relative rates of alternative pathways of pyrimidine metabolism.

Nucleotide concentrations. Concentrations of the major pyrimidine nucleotides in CHO cells are reported in Table 1. The concentration of UTP is 40 times that of dTTP,

TABLE 1. Pyrimidine Ribo- and Deoxyribonucleotide Concentrations in CHO-K1 Cells

Nucleotide	Concentration (pmoles/10^6 cells)
UTP	2,390
CTP	1,230
dTTP	58
dCTP	223

while that of CTP is only 5.5 times that of dCTP. dCTP is the largest deoxynucleoside triphosphate pool in most cell lines, but it is unusually large in these cells (review:3). It is the only deoxyribonucleotide which is detectable during HPLC measurement of the ribonucleotide pools (although HPLC was not routinely used to measure this pool).

Radioactive precursor studies. CHO cells were incubated with various radioactive precursors, samples were taken at different times up to 90 min, and the radioactivity in individual acid-soluble and RNA and DNA nucleotides was determined. Linear rates of incorporation into nucleic acids indicated that the flow through intermediate metabolites had reached a steady state; this required ca. 1 min for thymidine, less than 5 min for deoxyuridine, and approximately 20 min for uridine, cytidine and deoxycytidine. Most of the data presented here are from 30 to 40 min incubations, and incubation for longer periods ensured that the labeling rate did not decline due either to radiation effects or to depletion of precursor.

The main assumption of this work was that the addition of labeled precursors did not significantly perturb the cell's metabolism. That this is reasonable is indicated by observations that 10 to 100 times the concentration of cold precursor were required to cause measurable changes in nucleoside triphosphate concentrations (Henderson, J.F., unpublished). Although high specific activities (19 to 50 Ci/mmol) were used, no toxic effects were observed during the short term incubations.

Table 2 shows the results of 30 min incubations of CHO cells with tritiated uridine and cytidine, 40 min incubations with tritiated deoxycytidine and deoxythymidine, and a 65 min incubation with deoxyuridine. The data given in Table 2 represent the raw data from which other results were calculated.

In Table 3, the total conversion of each precursor to nucleotides has been calculated as the sum of radioactivity in acid-soluble and nucleic acid nucleotides (as reported in Table 2). This total in turn is divided into the total radioactivity (for each precursor) in the acid-soluble nucleotides, in RNA, and in DNA. (Any radioactivity in nucleosides and bases, which would be mostly in the medium rather than within the cells, was not measured.)

Table 3 also gives the specific activities of each of the precursors used in these experiments. By comparing these values with the values for the total conversion of radioactivity to nucleotides, it is possible to estimate the relative efficiencies of utilization of these precursors. For example, although the specific activities of the radioactive uridine and cytidine were identical, almost 4-fold more uridine than cytidine was converted to nucleotides during the 30 min incubation. Both compounds are phosphorylated by the same enzyme (4). The concentrations of uridine and cytidine were 0.5 μM and 0.3 μM, respectively.

TABLE 2. Incorporation of Radioactive Precursors into Pyrimidine Nucleotides

Nucleotides	Incorporation (counts/min per 10^6 cells)				
	Precursors				
	[6-^{3}H]Uridine	[5-^{3}H]Cytidine	Deoxycytidine	Deoxyuridine	Thymidine
UTP	438,000	934			
UDP	17,100	*			
UMP	8,300	*			
UDP-Glucuronic Acid	10,800	*			
UDP-Glucose	94,300	*			
CTP	125,000	147,000			
CDP	2,450	2,550			
CMP	*	2,360			
dTTP	5,300	+		149,000	665,000
dTDP	*	+		4,260	36,200
dTMP	*	+		3,862	13,100
dUMP	*	*		*	

(... cont. Table 2 ...)

Nucleotides	Incorporation (counts/min per 10^6 cells)				
	Precursors				
	[6-^{3}H]Uridine	[5-^{3}H]Cytidine	Deoxycytidine	Deoxyuridine	Thymidine
dCTP	32,000	35,800	69,400		
dCDP	3,510	600	4,000		
dCMP	*	523	7,176		
RNA-U	19,300	214			
RNA-C	5,220	10,500			
DNA-T	10,543	+		1,830,000	912,000
DNA-C	3,150	2,110	20,000		

[*] Trace.

[+] Radioactivity lost during conversion to this compound.

TABLE 3. Metabolism of Radioactive Pyrimidines in CHO-K1 Cells

Precursor	Specific activity	Sampling time	Total conversion to nucleotides	Amount in acid-soluble nucleotides	Amount in RNA	Amount in DNA
	(Ci/mmol)	(min)	(counts/min per 10^6 cells)			
Uridine	20	30	775,000	737,000	24,500	13,500
Cytidine	20	30	203,000	190,000	10,700	2,110
Deoxycytidine	20	40	101,000	80,600	0	20,000
Deoxyuridine	20	65	1,980,000	157,000	0	1,830,000
Thymidine	50	40	1,630,000	715,000	0	912,000

When corrected for differences in specific activity and incubation time, deoxyuridine was the most efficiently used of any of the precursors, including thymidine. Both deoxyuridine and thymidine are phosphorylated by the same enzyme (4). The concentrations of deoxyuridine and thymidine were 0.3 μM and 0.2 μM, respectively. This argues against thymidylate synthetase being rate limiting for thymidine nucleotide synthesis under these conditions. Deoxycytidine was converted to nucleotides much less efficiently than uridine, deoxyuridine and thymidine, and about half as efficiently as cytidine.

Figs. 1 to 3 express the amount of radioactivity that has flowed through a given reaction as a percentage of the total amount of radioactivity that was converted to nucleotides.

This way of presenting the data will be illustrated by considering the metabolism of UDP as an example. When radioactive uridine was the precursor, the total incorporation into nucleotides was 775,000 counts/min per 10^6 cells. Of these, 767,000 counts/min per 10^6 cells or 99% flowed into the UDP pool, while 744,000 counts/min per 10^6 cells or 96% flowed out, leaving 3% of the radioactivity in the UDP pool. Of the radioactivity that flowed through this pool, 729,000 counts/min per 10^6 cells or 94% was phosphorylated, while 15,500 counts/min per 10^6 cells or 2% was reduced by ribonucleotide reductase. Thus the ratio of reduction of UDP to phosphorylation is 2% to 94% or 1

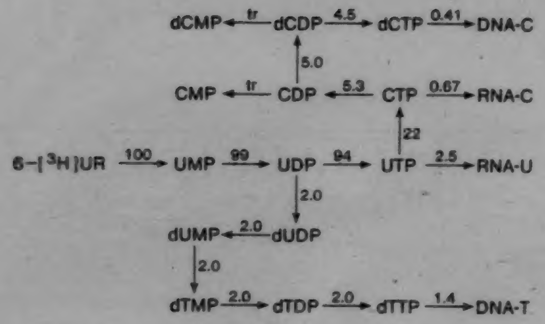

FIG. 1. Metabolism of [6-^{3}H]uridine. The amount of radioactivity from the precursor that has flowed through a given reaction (expressed as a percentage of the total amount of radioactivity converted to nucleotides) is given on the arrows representing individual reactions.

to 47.

In the analysis of these results, particular attention will be paid to the partition of radioactive metabolite flow at branch points in the pathways. Since DNA and RNA are "sinks" for radioactive label (review:5), longer incubations will increase the proportion of radioactivity present in nucleic acids without affecting the absolute amount in the acid-soluble nucleotides or the relative amounts in individual nucleotides. A significant amount of turnover of the radioactivity incorporated into RNA occurs, nevertheless RNA is still a net sink for radioactivity (6).

Uridine metabolism. The uridine was labeled in the 6 position; therefore, the label was not lost during the conversion of uridine nucleotides to deoxythymidine nucleotides (Fig. 1) and all the uridine, cytidine, deoxycytidine and thymidine nucleotides were labeled. Table 2 gives values for UDP-glucuronic acid and UDP-glucose, but for ease of illustration these compounds are not shown in Fig. 1. Since UTP is the precursor for these compounds, the values for the flow of label into them were included in the UTP value.

22% of the total flow of label into nucleotides was into the cytidine nucleotides. One would not expect a significant flow through the CMP pool under these conditions though CMP can be dephosphorylated to cytidine which in turn can be deaminated to uridine. This would not have been detected in this experiment; however, the deamination of cytidine was measured in other experiments and was found to be very low (Fig. 2).

The other branch point of interest is the conversion of UDP to deoxyribonucleotides; this reaction proceeded at ca. 1/47th the rate of the alternative pathway, phosphorylation to UTP. Of the total amount of radioactive CTP that was converted to CDP, a large percentage was converted to deoxyribonucleotides.

When the nucleotide pools have reached their steady-state specific activity, the amount of label in the pools can be used as a measure of the actual pool size as long as no dilution has occurred because of input of unlabeled precursor into the system from, for example a de novo pathway. Therefore, one would expect the ratio of the amount of radioactivity in UTP to CTP, which was 3.5 to 1 (Table 2), to reflect the ratio of the pool sizes as measured by HPLC (Table 1), which was 2 to 1. This agreement is fair considering that the ratios were determined by different methods and in separate experiments. The ratio of radioactivity in UTP to dTTP was 83 to 1, while the ratio of the pool sizes (Table 1) is one-half this value. This may indicate that the steady-state specific activity of the dTTP pool is lower than that of the UTP pool. The ratio of the radioactivity in CTP to dCTP was 4 to 1, which agrees with the ratio of the pool sizes which was 5.5 to 1.

Cytidine metabolism. The cytidine was labeled in the 5-position, and as a consequence the radioactivity was lost

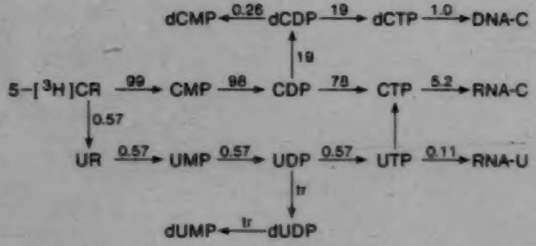

FIG. 2. Metabolism of [5-^{3}H]cytidine.

during thymidine nucleotide synthesis from dCMP and dUMP. 99% of the labeled nucleotides were present as cytidine nucleotides, indicating that cytidine deaminase activity was very low in these cells.

The incorporation of radioactivity from cytidine into CTP is an example of the very rapid equilibration of radioactivity among families of nucleotides. Since the cells are grown in nucleoside-free medium, the net synthesis of cytidine compounds follows the pathway UTP → CTP → CDP → dCDP. Nevertheless, when cells were incubated with radioactive cytidine, not only were both CDP and CTP rapidly labeled, but the radioactivity was predominantly in the triphosphate. As shown in Fig. 2, the label incorporated from cytidine into uridine nucleotides can be recycled into cytidine nucleotides via CTP synthetase. Therefore, the true value for the flow of cytidine into uridine nucleotides must be approximately 22% higher than the measured value of 0.57%, since, as shown in Fig. 1, 22% of the labeled nucleotides arising from [^{3}H]uridine as the precursor are converted to cytidine nucleotides.

The ratio of radioactivity in CTP to dCTP was 4 to 1 which is in reasonable agreement with the ratio of the pool sizes (Table 1) which was 5.5 to 1. The ratio of radioactivity in dCTP → dCDP → dCMP of 68 to 1.1 to 1 (Table 2) should equal the ratio of their pool sizes. Since the dCTP pool is 223 pmoles/10^6 cells (Table 1), the dCDP pool and the dCMP pool should be 3.6 and 3.3 pmoles/10^6 cells, respectively.

Deoxycytidine metabolism. Deoxycytidine is converted to deoxycytidylate by uridine-cytidine kinase (Fig. 3). The fact that these cells readily incorporate deoxycytidine into DNA will be useful for measuring DNA synthesis under conditions where thymidine kinase is inhibited, for example, by elevated deoxythymidine triphosphate. The ratio of radioactivity in dCTP to dCDP to dCMP was 9.6:0.6:1 (Table 2).

The deoxycytidine was labeled in the 5-position and therefore the radioactivity was lost in the conversion of dUMP to dTMP. Thus, it was not possible to measure the rate of deoxycytidylate deamination by simply measuring the accumulation of radioactivity in thymidine nucleotides and in DNA. The amount of radioactivity in the deoxyuridylate pool is really only a reflection of the pool size and not the flow through the pool. Deoxycytidine labeled in the 6-position could not be purchased.

Deoxyuridine metabolism. Deoxyuridine is incorporated into thymidine nucleotides by thymidine kinase (Fig. 3) and thymidylate synthetase. The data in Fig. 4 are calculated from a 65 min incubation and therefore the proportion of label in DNA is very high even though the absolute amount of label in thymidine nucleotides was considerable (Table 2). The radioactivity in dUMP was very low and the chromatographic location of the dUDP spot was not determined,

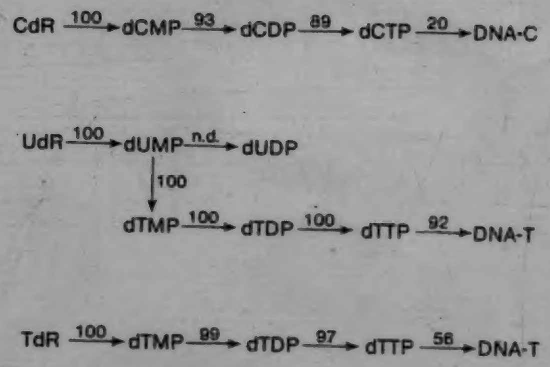

FIG. 3. Metabolism of radioactive deoxycytidine, deoxyuridine and thymidine.

although it probably separates from dTDP. The relative sizes of the thymidine nucleotide pools can be calculated from the ratios of their steady-state radioactivity. The ratio of radioactivity in dTTP to dTDP to dTMP was 38.5:1.1:1 (Table 2). Since the dTTP pool size is 58 pmoles (Table 1), the dTDP and dTMP pool sizes are calculated to be 1.7 and 1.5 pmoles, respectively.

Deoxythymidine metabolism. Deoxythymidine is metabolized by the same route as deoxyuridine except that it bypasses the thymidylate synthetase reaction (Fig. 3). The ratio of radioactivity in dTTP to dTDP to dTMP was 50.8: 2.8:1 (Table 2), and since the dTTP pool size was 58 pmole (Table 1), the calculated dTDP and dTMP pool sizes are 3.2 and 1.1 pmoles, respectively. These values are in agreement with those calculated with the [^{3}H]UdR.

Orotic acid metabolism. Although it has been reported that orotic acid is readily incorporated into nucleotides by some cultured cells, this was not the case with CHO cells. No radioactivity was detected in acid soluble nucleotides in CHO cells incubated 30 min with [^{3}H]orotic acid. This indicates that orotic acid either does not get into the cells, or that it is not used by the orotic acid phosphoribosyl transferase. Regardless of the cause, it is unfortunate that orotic acid is not metabolized because it would provide a good means of assessing the inhibition of the orotidylate decarboxylase reaction, which is inhibited by drugs such as pyrazofurin and 6-azauridine.

In conclusion, this study has provided considerable information on the metabolism of tracer concentrations of pyrimidines by CHO cells. The data have also been used to calculate certain pool sizes, such as dTMP, which would be difficult to measure by other means, and to estimate the relative efficiency of uptake of the various precursors.

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CHAPTER 6

MEASUREMENT OF RATES OF RNA AND DNA SYNTHESIS BASED ON THE SPECIFIC ACTIVITIES OF INTRACELLULAR RIBO- AND DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

INTRODUCTION

Measurement of relative rates of RNA or DNA synthesis, or both, by the incorporation of one or another radioactive precursor, involves the assumption that, when two or more experimental conditions are compared, no other parameter of precursor metabolism changes except the rate of nucleic acid synthesis. However, numerous drugs as well as physiological variables, may directly or indirectly alter either rates of conversion of precursors to the ribo- and deoxyribonucleoside triphosphates that are the immediate precursors of nucleic synthesis, or the concentrations and therefore the specific activities of the triphosphates. Knowledge of the specific activity of the actual precursor pools of nucleoside triphosphates is therefore necessary in order to calculate actual rates of nucleic acid synthesis, in contrast simply to relative rates.

Several investigators have called attention to these problems. As early as 1965, it was evident from the four-factor model for thymidine metabolism proposed by Stewart _et al_. (1) that changes in the relative rates of the salvage vs _de novo_ pathways would result in a change in the specific activity of the thymidine nucleotides, which in turn would alter the rate of incorporation of radioactivity into DNA. On the basis of experiments using [^{3}H]TdR and [^{3}H]CdR, Smets cautioned that incorporation rates could change markedly without corresponding changes in the actual rate of DNA synthesis (2). In addition, Injeyan et al. (3) found that growth inhibitory concentrations of hydroxyurea produced either stimulation or inhibiton of [^{3}H]TdR incorporation into DNA, depending on the hydroxyurea concentration. They concluded that the increase in [^{3}H]TdR incorporation was not a result of increased DNA synthesis. Other researchers have also concluded that incorporation of labelled precursors into RNA could not be directly equated with the rates of RNA synthesis (e.g., 4-6). In a few cases, nucleic acid incorporation data have been corrected for changes in the specific activity of the nucleoside triphosphate precursor (7), but this procedure has not been tested to determine if it actually measures the rate of nucleic acid synthesis.

We have attempted to develop a general method for the measurement of rates of RNA and DNA synthesis, which is independent of the particular radioactive precursor used and which corrects for changes in nucleotide concentrations or other aspects of purine or pyrimidine metabolism. This approach depends on the determination of the specific activity of each ribo- and deoxyribonucleoside triphosphate under conditions in which the flow of radioactivity through each triphosphate pool is in a steady state. An important assumption of this approach, which we have attempted to test, is that no compartmentation or metabolic "channelling" of nucleoside triphosphates occurs under the conditions used.

Finally, we have attempted to determine the rate of ribonucleotide reduction in intact cells from measurements of the actual rate of DNA synthesis.

MATERIALS AND METHODS

Sources of materials, and methods for the cultivation of CHO-K1 cells in alpha-MEM medium containing 10% dialyzed fetal calf seum, extraction of nucleotides, measurement of ribo- and deoxyribonucleotide concentrations, incubation with radioactive precursors, separation of individual acid-soluble ribo- and deoxyribonucleotides, and nucleotides or nucleosides from RNA and DNA, and the measurement of radioactivity, are described in preceding papers.

The method used to determine rates of DNA and RNA synthesis is based on the fact that when the rate of incorporation of radioactivity into RNA or DNA becomes linear in cells grown with radioactive precursors, the specific activity of the corresponding nucleoside triphosphate pool has reached a maximum; this is true even when the radioactivity is incorporated into the product macromolecule from two different precursors, e.g., ATP and GTP. The rate of DNA or RNA synthesis, in pmoles per min, is calculated by dividing the steady-state rate of incorporation of radioactivity into DNA or RNA, in counts/min, by the specific activity of the immediate precursor pool, in counts/min per pmoles.

RESULTS

Determination of the actual net rate of DNA or RNA synthesis requires correction of the results of simple incorporation data for dilution of the radioactivity by the intracellular nucleotide pools. This "specific-activity-corrected isotope incorporation" method involves determining the steady-state specific activity of the immediate precursor of DNA or RNA by measuring both the pool size of the nucleoside triphosphates and the amount of radioactivity in the pools. These measurements must be made under a steady-state condition, which is defined as the period when the rate of incorporation of radioactivity into DNA and RNA is linear, and therefore when the immediate precursors have reached their maximum specific activities. The values for the specific activities of the direct precursors of DNA or RNA, together with the DNA or RNA incorporation data allow the net rate of DNA or RNA synthesis, in pmole/min per 10^6 cells, to be calculated as described in the Methods section.

The results of measurements of the net rate of DNA synthesis by this method, using control CHO cells and a variety of radioactive precursors, are shown in Table 1. These data show that the results were not appreciably different when different precursors were used, or in different experiments. It was also established that when ribonucleoside precursors were used, the average steady state

Table 1

Rate of DNA synthesis in control CHO cells determined using specific-activity-corrected DNA incorporation data

Experiment	Precursor	Immediate DNA precursor	pmoles/min per 10^6 cells
			Rate of DNA synthesis
1	[6-^{3}H]uridine	dCTP	3.6
1	[6-^{3}H]uridine	dTTP	4.2
1	[5-^{3}H]cytidine	dCTP	4.4
1	[^{3}H]guanosine	dGTP	2.9
1	[^{3}H]adenine	dATP	1.2
1	[^{3}H]deoxycytidine	dCTP	2.0
1	[^{3}H]thymidine	dTTP	2.0
2	[6-^{3}H]uridine	dCTP	5.2
2	[6-^{3}H]uridine	dTTP	2.5
2	[5-^{3}H]cytidine	dCTP	4.0
2	[^{3}H]hypoxanthine	dATP	2.5
3	[^{3}H]deoxycytidine	dCTP	3.7
3	[^{3}H]thymidine	dTTP	3.4
4	[6-^{3}H]uridine	dTTP	1.4
4	[6-^{3}H]uridine	dCTP	3.2
		x =	3.1
		S.D. =	1.1
		S.E.M. =	0.30

specific activities of the deoxyribonucleoside triphosphates were equal to those of the corresponding ribonucleoside triphosphates (data not shown).

Two basic assumptions involved in the use of this method are (a) that the pool of each immediate precursor of DNA is homogenous, i.e., that there is no compartmentation or metabolic channeling, and (b) that newly synthesized DNA is not degraded. The first assumption is difficult to test directly; however, the fact that calculated rates of synthesis (Table 1) were very similar no matter which ribonucleoside or deoxyribonucleoside precursor was used, suggests either that there is no compartmentation or that any inhomogeneity affects all precursors equally.

In order to determine if the net rate of DNA synthesis was equal to the actual rate of DNA synthesis, it was necessary to determine the amount of degradation of newly synthesized DNA. Cells were incubated with [^{3}H]TdR for 7 min, and then resuspended in warm thymidine-free medium. Samples were removed for up to 1 h, centrifuged, the medium saved for radioactivity measurements, and the cell pellet extracted with perchloric acid. Both the total amount of acid-soluble and acid-insoluble radioactivity were determined. The results, shown in Fig. 1, demonstrate that once the radioactive precursor was removed, the radioactivity in the acid soluble fraction declined rapidly, as did the incorporation of radioactivity into DNA. After 20 min, the amount of radioactivity in DNA had stabilized, while

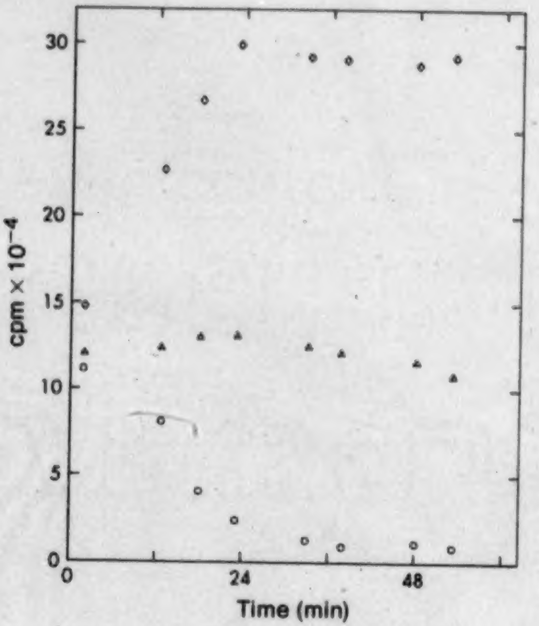

Figure 1. Temporal changes in the amount of radioactivity in DNA (◇) and in intracellular (○) and extracellular (△) acid-soluble thymidine compounds. CHO cells were incubated for 7 min with [^{3}H]thymidine, then collected by centrifugation and resuspended in thymidine-free medium. Samples were then removed for up to 1 h.

the amount in the medium did not change significantly during the entire chase. In a subsequent experiment it was found that the amount of radioactivity in DNA was stable for at least 3 h.

The specific activity corrected method was also tested to determine if it could be used to measure the rate of RNA synthesis. The control results shown in Table 2 demonstrate that while variation from precursor to precursor was small, the variation in the three experiments was substantial. Since the growth rates were approximately the same for the cells used in all the control experiments, and the rates of DNA synthesis were the same, there must be other factors causing the large variation in the apparent rate of RNA synthesis from experiment to experiment.

In order to determine if the net rate of RNA synthesis was equal to the actual rate of RNA synthesis, it was necessary to determine if significant degradation of newly synthesized RNA was occurring. Cells were incubated with [5-^{3}H]UR for 40 min, the radioactive precursor was removed by centrifugation and resuspension, and samples were taken at 30 or 40 min intervals up to 7 h. The net rate of loss of radioactivity from RNA was 0.1% of the total radioactivity in RNA per min; however, this value is not a measure of RNA turnover since recycling of the radioactivity probably occurs. These results show that significant RNA turnover does occur; thus, the total incorporation of radioactivity into RNA even when corrected for the specific

Table 2

Rate of RNA synthesis in control CHO cells determined using specific-activity-corrected RNA incorporated data

Experiment	Precursor	Immediate RNA precursor	pmoles/min per 10^6 cells Rate of RNA synthesis
1	[6-^{3}H]uridine	CTP	2.5
1	[6-^{3}H]uridine	UTP	6.4
1	[5-^{3}H]cytidine	CTP	4.8
1	[^{3}H]guanosine	GTP	7.0
1	[^{3}H]adenine	ATP	4.7
2	[6-^{3}H]uridine	CTP	22
2	[6-^{3}H]uridine	UTP	15
2	[5-^{3}H]cytidine	CTP	13
2	[^{3}H]guanosine	GTP	27
2	[^{3}H]hypoxanthine	ATP	18
2	[^{3}H]hypoxanthine	GTP	24
4	[^{3}H]uridine	UTP	22
4	[^{3}H]uridine	CTP	24
		Exp 1: x =	5.1
		S =	1.8
		Exp 2: x =	20
		S =	5.4

activity of the precursor is not a measure of the actual rate of RNA synthesis, but it may approximate the rate of stable RNA synthesis.

Having established the basic parameters of the specific-activity-corrected method, it was now necessary to compare the rates of nucleic acid synthesis obtained using it, with those obtained when this correction is not made.

To make this comparison, cells were treated with growth inhibitory concentrations of mycophenolic acid or pyrazofurin, and the apparent rates of DNA (Table 3) and RNA (Table 4) synthesis were determined by each method. These results demonstrate not only that values for the rates of DNA and RNA synthesis as determined by the simple isotope incorporation method depend on the radioactive precursor used, but also that with no precursor were the same results obtained as with the specific-activity-corrected incorporation method.

A further application of the method applied here, was to calculate the rate of synthesis of deoxyribonucleotides from ribonucleotides via ribonucleotide reductase, from the corrected rates of DNA synthesis from ribonucleoside precursors. In order for the rates of deoxyribonucleotide synthesis de novo to be calculated from the actual rate of DNA synthesis, the two assumptions that must be made are: 1) there must be no net synthesis of deoxyribonucleotides via salvage pathways and 2) there must be no net degradation of deoxyribonucleotides.

Table 3

Comparison of apparent rates of DNA synthesis during drug treatment determined by specific-activity-corrected isotope incorporation and by simple isotope incorporation measurements

Drug Treatment	Precursor	Rate of DNA synthesis; % of control	
		Specific-activity-corrected isotope incorporation	Simple isotope incorporation
Mycophenolic acid (2 μM; 2 hr)	[^{3}H]deoxyctyidine	21	88
Mycophenolic acid (2 μM; 2 hr)	[^{3}H]thymidine	17	5
Mycophenolic acid (2 μM; 2 hr)	[^{3}H]hypoxanthine	22	6
Pyrazofurin (0.2 μM; 2 hr)	[^{3}H]thymidine	48	100
Pyrazofurin (0.2 μM; 2 hr)	[^{3}H]adenine	42	33
Pyrazofurin (0.2 μM; 2 hr)	[^{3}H]deoxycytidine	30	580

Table 4

Comparison of apparent rates of RNA synthesis during drug treatment determined by specific-activity-corrected isotope incorporation and by simple isotope incorporation measurements

Drug Treatment	Precursor	Rate of RNA synthesis; % of control	
		Specific-activity-corrected isotope incorporation	Simple isotope incorporation
Mycophenolic acid (2 μM; 2 hr)	[^{3}H]hypoxanthine	23	10
Mycophenolic acid (2 μM; 2 hr)	[^{3}H]guanine	27	84
Mycophenolic acid (2 μM; 2 hr)	[^{3}H]uridine	30	2
Pyrazofurin (0.2 μM; 2 hr)	[^{3}H]uridine	52	111
Pyrazofurin (0.2 μM; 2 hr)	[^{3}H]cytidine	108	392
Pyrazofurin (0.2 μM; 2 hr)	[^{3}H]adenine	55	35

Assumption 1) was probably true since the cells were cultured in deoxyribonucleoside-free medium, but it was necessary to determine experimentally if assumption 2) was true. For example, it is possible that the rate of ribonucleotide reduction may normally be greater than the rate of DNA synthesis; the excess deoxyribonucleotides presumably would be degraded to deoxyribonucleosides or bases and be released into the medium. To measure deoxyribonucleotide catabolism, cells were pulsed with [5-^{3}H]CR or [6-^{3}H]UR for 30 min, after which the radioactive precursor was removed by centrifugation and resuspension. Samples were removed from the cell suspension for up to 1 h, centrifuged, the medium saved, and the cell pellet extracted with perchloric acid. The neutralized extract, as well as the hydrolyzed RNA and lyophilyzed medium, were analyzed by thin-layer chromatography.

No labelled deoxyribonucleosides were found in the cell extracts or in the medium. Since the chromatograms were oxidized to H_2O and CO_2, giving a tritium counting efficiency of 32%, small amounts of labelled deoxyribonucleosides would have been detected.

DISCUSSION

This study has provided direct evidence that the uncorrected rate of radioactive precursor incorporation into DNA and RNA is not a measure of their actual rates of synthesis. This was not a totally unexpected result since on the basis of indirect evidence, other researchers have cautioned against the use of this method (2,3).

It will now be necessary to re-evaluate results previously obtained with this method to determine if apparent inhibitions or stimulation of nucleic acid synthesis were simply a result of differences in the specific activities of the immediate nucleic acid precursors between control and drug treated cells, rather than actual differences in the rates of nucleic acid synthesis. For example, an apparent inhibition of DNA synthesis during drug treatment may simply have resulted from an inhibition of the phosphorylation of radioactive thymidine, or a stimulation of the *de novo* synthesis of thymidylate. This re-evaluation will be particularly important in cases where the main evidence for such drug-induced phenomenon as "unbalanced growth" was an apparent inhibition of DNA synthesis, as measured by uncorrected radioactive thymidine incorporation into DNA, coinciding with little or no inhibition of RNA synthesis, as measured by uncorrected radioactive uridine incorporation into RNA.

It was concluded that the specific-activity-corrected incorporation method measures the net rate of both DNA and RNA synthesis; however, the net rate of nucleic acid synthesis is equal to the total rate only if there is no significant degradation of newly incorporated nucleotides during the measurement period. Significant turnover of eucaryotic DNA has not been reported and our results confirmed that in untreated cells, newly incorporated nucleotides are stable. However, it is known that some types of RNA, mainly heterogeneous nuclear RNA, but also some ribosomal-precursor RNA, are degraded in some cell types (8); von Tigerstrom observed a rapid degradation of approximately 50% of the rapidly labelled RNA in Ehrlich ascites tumor cells in vitro (9). We also observed significant degradation of newly labelled RNA in CHO cells. Therefore, the total rate of RNA synthesis cannot be determined by the specific-activity-corrected incorporation method although the net rate of synthesis can be measured. If the different classes of RNA were separated, then the actual rates of synthesis could be determined for the classes in which the radioactive label was stable. For example, Emerson and Humphrey have done this in order to measure the rate of ribosomal RNA synthesis in fibroblasts. They labelled the cells with [^{3}H]adenosine and calculated an average specific activity for the ATP pool during the total incubation period. This value plus the total ribosomal incorporation allowed them to calculate a value for the rate of rRNA synthesis, with their measured value comparing well with a theoretical value based on the rRNA content of the cells and their generation time (10).

A major assumption of the specific-activity-corrected method is that the entire pool of a given direct precursor of DNA or RNA is freely mixing, i.e., that there is no compartmentation or metabolic channelling occurring. In view of the number of reports on compartmentation of nucleic acid precursors, this assumption cannot be taken for granted (11-17). Most recently, Reddy and Pardee have reported that in permeabilized Chinese hamster embryo fibroblast cells, ribonucleoside diphosphates were incorporated into DNA 25% faster than deoxyribonucleoside triphosphates. They concluded that metabolic channelling occurs, starting at ribonucleotide reductase, such that compounds entering the system at or before this step are incorporated in preference to compounds entering after this step (18).

We tested the assumption of homogenous nucleoside triphosphate pools in two ways.

First, the rate of DNA synthesis in untreated cells was measured using both radioactive ribonucleoside and deoxyribonucleoside precursors. Therefore, if as Reddy and Pardee have reported for permeabilized cells, preferential channelling of ribonucleotides into DNA occurs, a significant difference between the apparent rate of DNA synthesis as measured using ribonucleosides and deoxyribonucleosides would be expected. No such difference was observed in CHO cells. It should be noted that the difference in incorporation rates of ribonucleotides versus deoxyribonucleotides reported by Reddy and Pardee was only 20-25%, which is the minimum difference that could have been detected in our results.

In addition, when radioactive ribonucleoside precursors were used, the specific activity of each deoxyribonucleoside triphosphate and the corresponding ribonucleoside triphosphate precursor were compared. If compartmentation of the ribonucleotides were occurring, such that one pool was being used for deoxyribonucleotide synthesis and another was being used for RNA or, if as Reddy and Pardee suggest, most of the deoxyribonucleoside triphosphate pools in cells are not used for replicative synthesis, one would not expect the specific activities of the ribonucleoside and corresponding deoxyribonucleoside triphosphates to be equal. However, the average specific activity of the ribonucleoside triphosphate pools for all the experiments was equal to that of the deoxyribonucleoside triphosphate pools.

The final purpose of this study was to measure the rate of ribonucleotide reduction in intact cells. The approach used was to relate the rate of ribonucleotide reduction to the rate of DNA synthesis. Therefore it was necessary to determine the rates of deoxyribonucleoside catabolism and synthesis, via salvage pathways. Under control conditions it was found that no measurable DNA catabolism occurred. Therefore when CHO-K1 cells are cultured in deoxyribonucleoside-free medium, there is little or no flow from the salvage pathways into deoxyribonucleotide pools. Furthermore, under control conditions, no measurable net pyrimidine deoxyribonucleotide catabolism to bases or nucleosides occurred. Purine deoxyribonucleotide catabolism could not be measured by this method since both deoxyadenosine and deoxyguanosine are rapidly converted to ribonucleotides by these cells.

These results indicate that at least for the pyrimidine nucleotides, a simple equation can be written as follows: the rate of ribonucleotide reduction of a given nucleotide equals the rate of increase in the deoxyribonucleotide pool size plus the rate of DNA synthesis. Therefore, using the methods presented here, it is now possible to measure the rate of ribonucleotide reduction *in vivo* by measuring the rate of DNA synthesis and the rate of change in the precursor pools.

In order to measure the individual rates of UDP and CDP reduction, rather than their sum, it will be necessary also to determine the amount of deoxycytidylate reduction occurring in these cells.

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CHAPTER 7

MODELS OF RIBONUCLEOTIDE REDUCTASE REGULATION AND THEIR EVALUATION IN INTACT MAMMALIAN CELLS

INTRODUCTION

Models of the structure, mechanism of catalysis and regulation of ribonucleotide reductase (ribonucleoside diphosphate reductase, EC 1.17.4.1), based on enzymological studies, have been extremely influential since 1966 in discussions and investigations of deoxyribonucleotide metabolism, deoxyribonucleoside toxicity, and the regulation of DNA synthesis by the supply of substrates. As studies of this enzyme have progressed, new models of ribonucleotide reductase and its regulation have been published. As is to be expected, these models have been identical or similar in some respects, and many investigators have in practice tended to view them all as identical or interchangeable. A more detailed study of these models, however, reveals some very significant differences among them, differences which ought to affect the interpretation of studies of deoxyribonucleotide metabolism and deoxyribonucleoside toxicity in intact cells. This review considers the ten major models of ribonucleotide reductase action and regulation, and indicates both their similarities and differences.

A relatively small number of studies have also been carried out to determine how the reduction of ribonucleotides is regulated in intact mammalian cells, and these usually have been influenced by or related to one or another model of ribonucleotide reductase. These investigations will also be reviewed here, and the present state of this question will be summarized.

MODELS OF RIBONUCLEOTIDE REDUCTASE REGULATION

Nine of the ten models to be described were based in whole or in part, on kinetic studies. One, however, was based entirely on binding and other physiochemical measurements, and such data entered into some of the other models either explicitly or implicitly.

Most investigators have presented the results of their studies of ribonucleotide reductase in terms of various schemes or drawings; these interpretations are what are here called "models". It is important to note, however, that in most cases additional pertinent data were reported in the various publications, but not incorporated into the models themselves.

For the present purposes, we have summarized all of the models based on kinetic studies in a uniform, tabular format (Tables 1-9). The schemes based on binding and other physiochemical studies, however, require a separate format (Figs. 1-5). In the accompanying text, in each case, other important information given in each study will be summarized as well.

Model 1

In 1966, Moore and Hurlbert (1) published the first model depicting the control of ribonucleotide reductase, based on studies of the partially purified enzyme from

Novikoff rat hepatoma cells. Their model is summarized in Table 1.

Other findings which are either not obvious from the model or which were not included in the model are as follows:

1) dTTP was required for GDP reduction to occur. The rate of reduction in the absence of dTTP was less than 10% of the rate in the presence of dTTP.

2) Either dGTP or dTTP was required for ADP reduction to occur, with dGTP being much more effective. In the absence of activator, no reduction occurred.

3) 1 to 2 mM ATP was required in order for reduction of pyrimidines to occur. The rate of reduction without ATP was less than 4% of the rate with ATP.

4) The inhibition of CDP reduction by dATP or dGTP was reversed by ATP.

5) ATP was reduced less rapidly than ADP. At equal concentrations, CDP was reduced 30% faster than CTP.

6) The enzyme preparation contained nucleoside diphosphate kinase activity, which had the effect of lowering substrate concentrations when nucleoside triphosphates, especially ATP, were present. No phosphatase activity was detected.

Model 2

In 1966 Larsson and Reichard (2) published the results of kinetic studies with partially purified ribonucleotide reductase from *E. coli*. The enzyme was essentially free of

Table 1

Model 1: Moore & Hurlbert (1966)

Substrate	Effects of Nucleotides				
	dTTP	dCTP	dGTP	dATP	ATP
CDP	I	0	I^1	I^1	S^R
UDP	I	0	I	I	S^R
GDP	S^R	0	I	I	S
ADP	S^R	0	S^R	I	0

[1] Inhibition competitively reversed by ATP.

I = inhibitory

S = stimulatory

0 = no effect

R = required

pyrophosphatase and kinase activities. Many of their findings are summarized in the two schemes shown in Fig. 1 and Table 2. Figure 1 depicts a model for the conformational changes induced in ribonucleotide reductase by allosteric effectors, while Table 2 represents Reichard's first attempt to suggest how his enzymological findings might be physiologically significant.

Reichard did not state that this model applies to mammalian cells, but did say that "... a striking parallelism is observed with the results of Moore with enzymes from Novikoff hepatoma".

Other results of this study which should be noted are as follows:

1) ATP stimulated CDP and UDP reduction, but the rate of reduction in the absence of ATP was approximately 20% of the rate in this presence.

2) dTTP stimulated CDP and UDP reduction in the absence of ATP, but inhibited their reduction in the presence of ATP.

3) dGTP, in the presence of ATP, inhibited CDP and UDP reduction. This result was not included in the model and was not studied further although it agrees with the results of Moore and Hurlbert.

4) UTP was the only ribonucleoside triphosphate which gave a stimulation of GDP reduction comparable to the stimulation produced by dTTP or dGTP. However, the maximum stimulation by UTP was at 1 mM, which was 100 times the

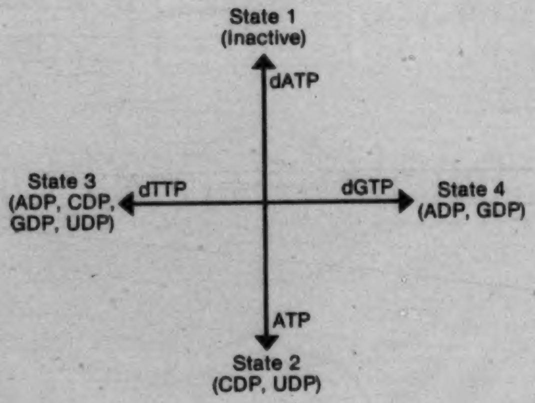

FIG. 1. Model of Larsson and Reichard (1966; ref. 2). Schematic interpretation of allosteric effects on ribonucleotide reduction.

Table 2

Model 2: Larsson & Reichard, 1966

Substrate	Effects of Nucleotides				
	dTTP	dCTP	dGTP	dATP	ATP
CDP	S (ATP$^-$) I (ATP$^+$)	0	0	I	S^r
UDP	S (ATP$^-$) I (ATP$^+$)	0	0	I	S^r
GDP	S	0	S	I	O
ADP	S	O	S	I	O

r = partially required

concentration of dTTP or dGTP required for maximum stimulation. (These findings also were not included in the model, although we have found that the concentration of UTP in cultured CHO cells is ca. 2.4 mM.)

5) Evidence was obtained that different types of binding sites might exist for ATP and dTTP.

6) Some of the data strongly suggested that one enzyme catalyzed the reductions of all four substrates.

7) UDP was a competitive inhibitor of CDP reduction and vice-versa. This result was obtained in the presence of ATP which, according to the model, should make the enzyme specific for pyrimidine nucleotide substrates ("pyrimidine specific").

8) In the presence of dTTP, GDP competitively inhibited CDP reduction, even though the model predicts that dTTP would make the enzyme GDP specific. In the presence of ATP, which is predicted to make the enzyme pyrimidine specific, GDP inhibited CDP reduction less than in the presence of dTTP. According to the model, GDP and CDP are reduced by different allosteric states of the enzyme; therefore, one would not expect them to compete for reduction.

9) The enzyme did not reduce GMP, and was 5% as active with GTP as with GDP.

Model 3

In 1969, Brown and Reichard (3) published the results of binding studies with purified ribonucleotide reductase from E. coli; no kinetic studies were included. They incorporated many of the results of their study into the model shown in Figure 2.

This model is considerably more elaborate than the one presented in 1966 (Table 2), which was formulated without the aid of binding data. Although other data had suggested the existance of different types of allosteric binding sites, this was the first time that good evidence for their existance was presented. For the sake of simplicity the scheme presented (Figure 2) shows only one l-site and one h-site per molecule, but in fact there were two per molecule. The term "h-site" refers to a "high affinity site", while "l-site" refers to a "low affinity site" for the binding of dATP. The dissociation constant of dATP from the h-site was 50 nM while the value for the l-site was 210 nM.

Other results which should be noted are as follows:

1) h-Sites had a high affinity for dATP, but also bound dTTP, dGTP and ATP.

2) The removal of one-half the dATP bound to the h-sites required a 20-fold excess of dTTP or a 5000-fold excess of ATP.

3) l-Sites had a low affinity for dATP, some affinity for ATP and no affinity for dTTP or dGTP.

4) The enzyme was relatively inactive in the absence of effectors, in agreement with Moore and Hurlbert's results with Novikoff hepatoma ribonucleotide reductase (1).

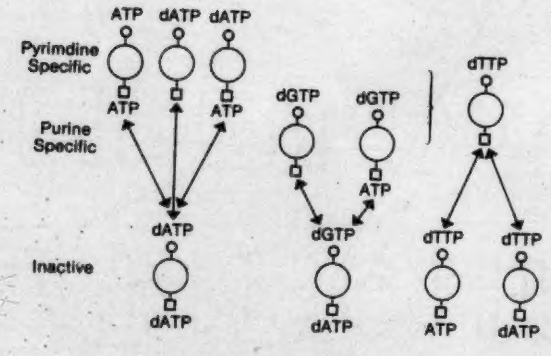

FIG. 2. Model of Brown and Reichard (1969; ref. 3). Scheme for different forms of protein B1 of ribonucleotide reductase. The h-sites (specificity sites) are represented by circles, the l-sites (activity sites) by squares. For convenience only one site of each class is depicted. The arrows show transitions between active and inactive forms.

This differs from Reichard's opinion in 1965 when he stated "The ATP requirement was not absolute and was more pronounced at the lower CDP concentration" (4). This difference may only be a matter of degree, since no numbers were presented.

5) With one exception, all the inhibited states of the enzyme had dATP bound to the l-sites. The exception occurred when dTTP was bound to the h-sites and ATP to the l-sites. In contrast, the model of Moore and Hurlbert indicates that this state would preferentially reduce GDP, since they found that dTTP stimulated GDP reduction in the presence or absence of ATP, although less dTTP was required for maximum stimulation in the absence of ATP.

6) The data indicate that the l-sites probably regulate the level of activity of the enzyme, while the h-sites regulate substrate specificity.

Model 4

In 1976, von Döblen and Reichard (5) published further binding studies with ribonucleotide reductase from *E. coli*, this time dealing with substrate binding; some kinetic experiments were also done. The study was complicated by the fact that the reductase preparation was contaminated with kinase activity which, in the presence of nucleoside triphosphates, converted the added substrates to triphosphates. Therefore, various nucleoside triphosphate analogues were used as allosteric effectors. In kinetic studies with the reductase it was found that these analogues affected the reaction in ways similar, but not identical, to the natural effectors. Thus it was found that a 10-fold higher concentration of dAMP-P-(CH_2)-P could substitute for dATP, a 6-fold higher concentration of dTMP-P-(CH_2)-P could substitute for dTTP, and a 50-fold higher concentration of dGMP-P-(NH)-P or a 3-fold lower concentration of dGMP-P-P-(S) could substitute for dGTP. None of the analogues tested could substitute for ATP. A second complication was that in order to prevent utilization of the substrates, only the B1 subunit was used in the detailed binding studies. Subunit B2 was found to bind neither substrates nor effectors, while B1 bound both, but showed no catalytic activity by itself. The use of the B1 subunit alone brings into question the relevance of the results both for the holoenzyme and for the whole cell, especially since von Döblen and Reichard concluded that the catalytic site is constructed from B1 and B2 together.

The major findings of this study are described below, and the results of the kinetic studies are summarized in Table 3.

1) The dTTP analogue and the dGTP analogue each lowered the dissociation constant (K_{diss}) for the binding of GDP to subunit B1, but neither effector competed with GDP for binding. These conditions stimulated GDP reduction. Low concentrations of the dATP analogue did not affect GDP reduction and at higher concentrations, which strongly

Table 3

Model 4: von Döblen and Reichard, 1976

Substrate	Effectors			
	dTTP	dCTP	dGTP	dATP
CDP	S	[2]	S[1]	S
UDP	S	[2]	0	S
GDP	S	[2]	S	I
ADP	S	[2]	S	I

These experiments were performed in the absence of ATP or an ATP analogue.

[1] Effect on enzyme activity, but not substrate binding, was measured.

[2] not included in model.

S = stimulation of binding and enzyme activity

I = inhibition of binding and enzyme activity

O = no effect

inhibited GDP reduction, GDP binding was only slightly decreased.

2) The dATP analogue and the dTTP analogue each lowered the K_{diss} for the binding of CDP to subunit B1, and also stimulated the reduction of CDP.

3) At low concentrations the dTTP and the dGTP analogues each increased the binding of ADP to subunit B1 (i.e., the K_{diss} was lowered). These conditions also stimulated ADP reduction. The dATP analogue had no effect on ADP binding at low or high concentrations. This is not consistent with the model of Brown and Reichard (Model 3) which predicts that low concentrations of dATP will bind to the specificity sites and made the enzyme pyrimidine specific, whereas high concentrations will bind to both the specificity and activity sites and inhibit the enzyme completely.

4) No S-shaped binding curves were observed indicating lack of cooperativity for the binding of allosteric effectors.

5) CDP and GDP competed for the same sites on B1. This result agrees with that of Larsson and Reichard (2) in 1966. These experiments were carried out in the presence of the dTTP analogue which should have converted the enzyme to a GDP specific state. It has been demonstrated that the enzyme is inactive in the absence of a positive effector; therefore, the dTTP analogue must be bound to the enzyme but it raises the question why CDP can bind to the enzyme if it is GDP specific.

6) It was not possible to demonstrate simple competition between ADP and GDP although the presence of one substrate decreased the binding of the other.

7) The Scatchard plots indicated two binding sites each for CDP and GDP, but the plot suggested at least 4 binding sites for ADP, although it is possible that the ADP was binding to some effector sites as well as substrate sites.

Model 5

Reichard published the next model for the regulation of ribonucleotide reductase in 1978, in a review (6). The essential ingredients of the model were described using the following three schemes: Figure 3 depicts the physical make-up of the enzyme, showing the subunit construction and the location of the allosteric effector binding sites and of the substrate binding sites. Figure 4 shows the different allosteric forms of the *E. coli* reductase, while Table 4 shows the model presented for the regulation of ribonucleotide reduction in cells.

It is important to note the differences between the model presented in 1969 (3) and the one presented in 1978. Substantial changes occurred over that period although no data were presented to explain the changes. The differences in the models are as follows.

1) In the 1969 model (Figure 2), the l-sites (activity sites) could either be occupied by ATP or dATP, or left

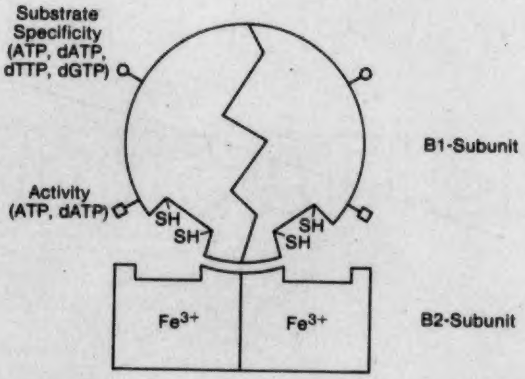

FIG. 3. Model of Reichard (1978; ref. 6). Model of *E. coli* ribonucleotide reductase.

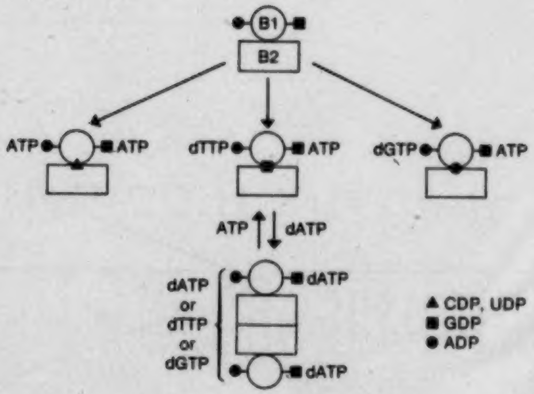

FIG. 4. Model of Reichard (1978; ref. 6). Main allosteric forms of ribonucleotide reductase. Binding of ATP to "activity sites" (■) results in catalytically active forms of the enzyme. The substrate specificity of these forms is determined by binding of effectors to "specificity sites" (●). Binding of dATP to "activity sites" gives an inactive dimer.

Table 4

Model 5: Reichard, 1978

Substrate	Effects of Nucleotides				
	dTTP	dCTP	dGTP	dATP	ATP
CDP	I	O	O	I	S
UDP	I	O	O	I	S
GDP	S	O	O	I	S
ADP	O	O	S	I	S

unoccupied. All the states with the l-sites unoccupied were active. In the 1978 model the l-sites were always occupied, either by ATP or dATP.

2) In the 1969 model, dATP, ATP, dGTP, or dTTP completed for the h-sites (specificity sites) on the active forms of the enzyme. In the 1978 model, dATP was not included as a competitor for the h-sites on the active forms of the enzyme, but was included as an h-site competitor on the inactive form of the enzyme although it was not really important which effector occupied the specificity sites on the inactive form of the enzyme. The omission of dATP as a competitor for the specificity sites on the active enzyme is inconsistant with the data which shows that dATP competes well for the h-sites; for example, the K_{diss} for dATP was 50 nM, and for dTTP was 300 nM (3). As well, in the 1969 model occupation of the h-site by dATP caused the enzyme to reduce pyrimidines specifically.

3) The final difference in the models involves the activity of the enzyme when dTTP occupies the h-sites and ATP occupies the l-sites. In the 1969 model this form of the enzyme was inactive, whereas in the 1978 model this form specifically reduced GDP.

One further point concerning the 1978 model is that Reichard used it to explain the results of experiments in mammalian cells in which deoxyribonucleotide pool size changes had occurred, even though the model developed from experiments with *E. coli* ribonucleotide reductase.

One possible explanation for the differences between the 1969 and the 1978 models is that the 1978 model may have been influenced by the results of experiments using intact cells. For example, using the 1969 model, one would predict that an increase in dTTP in intact cells could cause either an increase or a decrease in dCTP, depending on the ATP concentration. However, in 1973 Bjursell and Reichard (7) published results demonstrating that treatment of Chinese hamster ovary cells with thymidine resulted in an 18-fold increase in dTTP and a 30-fold decrease in dCTP. Perhaps results such as these influenced Reichard to modify his model to conform more closely to the available intact cell data.

Model 6

In 1979 Eriksson, Thelander and Akerman (8) published the results of a study of the allosteric regulation of calf thymus ribonucleotide reductase. This was the first thorough study of the regulation of a purified mammalian ribonucleotide reductase since the work of Moore and Hurlbert in 1966 (1). The enzyme preparation used in Eriksson *et al*.'s study contained no detectable phosphatase activity and only trace amounts of kinase activity.

Their model is summarized in Table 5, and the main results of their study are as follows.

1) The reduction of ribonucleotides required the presence of a positive allosteric effector. No deoxyribonucleotide formation could be detected in the absence of an

Table 5

Model 6: Eriksson, Thelander & Akerman, 1979

Substrate	Effects of Nucleotides				
	dTTP	dCTP	dGTP	dATP	ATP
CDP	I	O	I	I	S
UDP	I	O	I	I	S
GDP	S	O	I	I	S*
ADP	O	O	S	I	S

*ATP was an activator only in the presence of dTTP.

effector, even with high ribonucleotide concentrations.

2) The reduction of CDP specifically required ATP, with optimal concentrations of 2 to 5 mM. Other triphosphates, e.g., dTTP, dGTP, dATP and dCTP, did not substitute for ATP.

3) In the presence of 1 mM ATP, CDP reduction was inhibited by dATP, dTTP, and dGTP, but not by dCTP. dATP was the most effective inhibitor, while inhibition by dTTP and dGTP was not evident at high CDP concentrations, but became pronounced at CDP concentrations close to the K_m (30 μM). (We have found that the actual concentration of CTP in CHO cells is 1.2 mM, and the concentration of CDP is 0.03 to 0.1 fold that of the CTP.)

4) The reduction of UDP required ATP; again, optimum concentrations were 2 to 5 mM.

5) In the presence of 1 mM ATP, UDP reduction was inhibited 50% by 5 μM dATP or by 100 μM dTTP or dGTP.

6) Reduction of GDP required dTTP while other triphosphates (e.g., dATP, dGTP or dCTP) had no effect as positive effectors. (In the presence of dTTP, ATP was an activator.)

7) GDP reduction was inhibited 50% by 1 μM dATP, in the presence or absence of dTTP, or by 50 μM dGTP. (We have found that the actual concentration of dGTP in CHO cells is ca. 10 μM.)

8) The reduction of ADP specifically required dGTP. ATP, dATP, dTTP and dCTP showed no effect as positive effectors. dGTP plus ATP gave a 2-fold higher activity than

dGTP alone.

9) ADP reduction was inhibited 50% by 50 μM dATP in the presence of dGTP plus ATP, or by 300 μM dTTP. These results differ from Moore and Hurlbert's results with Novikoff hepatoma reductase in which dTTP stimulated ADP reduction. Reichard's 1966 model showed a stimulation of ADP reduction by dTTP but his 1978 model did not.

10) The presence of dTTP at 20 μM and ATP at 1 mM allowed the reduction of both CDP and GDP. This disagrees with Brown and Reichard's 1969 model in which these conditions produced an inactive enzyme. It also disagrees with Reichard's 1978 model in which these conditions produced a GDP-specific enzyme.

11) GDP and CDP competed for the same catalytic site. This experiment was carried out in the presence of dTTP and ATP which allowed reduction of both GDP and CDP. A Lineweaver-Birk plot showed competitive inhibition of CDP reduction by GDP. The fact that GDP and CDP competed for reduction is evidence that one enzyme state reduced both substrates, and not that two states existed, one specific for CDP and one for GDP.

12) The reductase was 10% as active with CTP as substrate as with CDP with each compound at 500 μM. However, we have found that in CHO cells the ratio of CTP to CDP is more than 10 to 1. Further study is required to establish the identitiy of the substrates for the reductase in vivo.

Eriksson *et al.*'s model is very similar to Reichard's 1978 model, although the following important difference should be noted: Reichard's model did not show inhibition of CDP, UDP or GDP reduction by dGTP, while Eriksson *et al.*'s did. It should also be noted that both models represent a selection of the data; for example, Eriksson *et al.*'s model did not include inhibition of ADP reduction by dTTP although they found this to occur.

Model 7

In 1979 Thelander and Reichard (9) published another model for the regulation of ribonucleotide reductase in intact cells. The model was presented in a review which contained little new data, yet it was different from Reichard's 1978 model and from Eriksson *et al.*'s 1979 model. The new model appears to be a composite of the other two models for *E. coli* and calf thymus ribonucleotide reductase. In fact the authors state "*In vitro*, the activity and substrate specificity of the reductases from *E. coli* and mammalian sources behave similar (sic) towards allosteric effectors. The data can be integrated into a scheme that links ribonucleotide reduction to DNA synthesis." It appears that this new model is meant to describe the behaviour of both *E. coli* and calf thymus ribonucleotide reductases. This model is shown in Figure 5 and Table 6.

Although most of the models for the regulation of

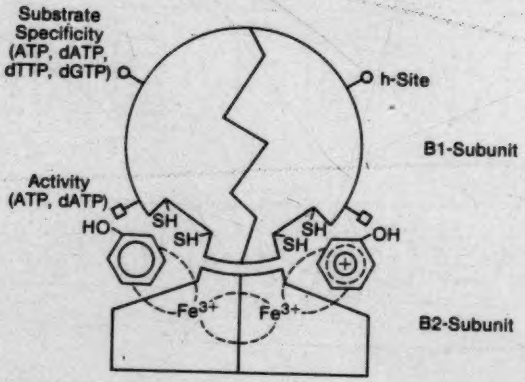

FIG. 5. Model of Thelander and Reichard (1979; ref. 9). Model of ribonucleotide reductase from E. coli.

Table 6

Model 7: Thelander & Reichard, 1979

Substrate	Effects of Nucleotides				
	dTTP	dCTP	dGTP	dATP	ATP
CDP	I	O	I	I	S
UDP	I	O	I	I	S
GDP	S	O	I	I	S
ADP	O	O	S	I	S

deoxyribonucleotide synthesis in vivo or in intact cells have come from Reichard's group, other models have been proposed which are based to varying degrees on either Reichard's models or Moore and Hurlbert's model. The contributors of these "second generation" models to the ribonucleotide reductase story are sufficiently important to justify dicussing their development separately from the evaluation of Reichard's models.

Model 8

One such model was proposed by Werkheiser *et al*. in 1973 (10) (Table 7). It was based very loosely on both the 1969 model of Brown and Reichard and the model of Moore and Hurlbert.

The following details of the model should be noted:

1) The model is an open steady-state system such that the concentrations of CDP, UDP and ADP are constant, and the product, DNA, is an irreversible sink.

2) The synthesis of dCTP, dTTP, dATP and dUMP is regulated by feedback inhibition of ribonucleotide reductase, which is represented as three separate enzymes.

3) The metabolism of guanine nucleotides is not included in the model.

4) Ribonucleotide reductase has a constant V_{max} for each substrate.

Obviously, there are large differences between this model and any of the models proposed by Reichard or Moore and Hurlbert. The major differences are as follows:

Table 7

Model 8: Werkheiser _et_ _al_., 1973

Substrate	Effects of Nucleotides					
	dUMP	dTTP	dCTP	dGTP	dATP	ATP
CDP			I			
UDP	I	I				
GDP	—	—	—	—	—	—*
ADP	I				I	

*Not included in model.

1) Werkheiser et al.'s model does not include stimulation of ribonucleotide reductase, either by deoxyribonucleotides or by ATP.

2) This model does not include the reduction of GDP, nor the effects of dGTP on other reductions.

3) This model includes only one interaction between different families of deoxyribonucleotides.

4) It includes a feedback inhibition role for dCTP and dUMP.

5) One final important difference among the models is that Werkheiser et al.'s model treats ribonucleotide reductase as three separate enzymes, and hence does not include an inhibition of reduction by substrate competition for a single catalytic site; the models of Reichard and Moore and Hurlbert include this feature.

Werkheiser et al. recognized these differences and stated that the true pattern of feedback inhibition was probably quite different from their model. Nevertheless, they used their model to predict the effect of pairs of drugs on DNA synthesis, and therefore on growth rate. All the drugs used were specific inhibitors of deoxyribonucleotide or DNA synthesis. They compared their results with the results obtained by Grindey and Nichol (11) in cultured L1210 cells, and concluded that "The over-all agreement is good and suggests that this model does in some sense mimic actual events in the intact cell." They also noted that the deoxyribonucleotide pool size changes predicted by their model agreed with some results of other researchers using intact cells. It is interesting that although this model is very simple and quite different from Reichard's models it still appears to mimic the situation in intact cells.

Nicholini et al. (12) have used Werkheister et al.'s model to analyze the effects of combinations of the antimetabolites, hydroxyurea, FUdR, ara-A, ara-C and methotrexate, on deoxyribonucleoside triphosphate concentrations. Their study was limited to computer modelling and unlike Werkheiser et al.'s study (10), did not include a comparison of the results generated by the computer model with results obtained in cultured cells.

Model 9

In 1975, Grindey, Moran and Werkheiser (13) published a much more complicated version of their previous model; this is summarized in Table 8. This model resembles more closely the models of Moore and Hurlbert and Reichard then did Werkheiser et al.'s 1973 model. The major differences between this new model and Reichard's models are as follows:

1) Grindey et al.'s model represents the reductase as four separate enzymes.

2) The model includes dCMP deaminase, which is stimulated by dCTP and inhibited by dTTP.

3) ATP does not act as a general activator of all reductions or as a specific activator of pyrimidine reduction.

Table 8

Model 9: Grindey, Moran & Werkheiser, 1975

Substrate	Effects of Nucleotides					
	dUMP	dTTP	dCTP	dGTP	dATP	ATP
CDP	I	I	O	I	I	O
UDP	I	I	O	I	I	O
GDP	O	S	O	I	I	O
ATP	O	O	O	O	I	O

As with Werkheiser et al.'s 1973 model, this model was used to predict the effects of pairs of drugs on DNA synthesis and thus on cell growth. The results compared very well with the results obtained in cultured L1210 cells by Grindey and Nichol (11). In a few cases this new model predicted drug effects better than Werkheiser's original model, but in most cases there was little room for improvement. Therefore, both very simple and more complicated models could be consistent with data from intact cells.

Model 10

The most comprehensive mathematical model of cellular metabolism to date has been developed by Jackson (14). The entire model includes 63 reactions which have been grouped under the headings of "folate dependent reactions", "reactions of nucleotide metabolism" and "reactions involving membrane transport and activation of drugs". The portion of the model dealing with ribonucleotide reduction is summarized in Table 9. This part of the model is based on the model of Moore and Hurlbert, although the following differences should be noted:

1) Jackson included one result of Moore and Hurlbert which they had not included in their model. That is, Jackson defined low and high concentrations of dTTP as activating and as inhibitory, respectively, for GDP reduction, whereas Moore and Hurlbert included dTTP only as an activator.

Table 9

Model 10: Jackson, 1980.

Substrate	Effects of Nucleotides				
	dTTP	dCTP	dGTP	dATP	ATP
CDP	I	O	I	I	O
UDP	—	—	—	—	—*
GDP	S/I[**]	O	I	I	O
ADP	S	O	S	I	O

[*] Not included in model.

[**] dTTP was defined as an activator at low concentrations and an inhibitor at high concentrations.

2) Jackson represented ribonucleotide reductase as three separate enzymes, thus omitting substrate competition, whereas Moore and Hurlbert included substrate inhibition.

3) Jackson omitted UDP reduction as a source of dTTP. All dTTP was thus derived via dCMP deamination.

4) He did not include ATP either as a general activator of all reductions or as a specific activator of pyrimidine reduction.

5) One final point which clearly distinguishes this model from either the Moore and Hurlbert model or the Grindey _et al_. model, is that GDP and ADP reduction obey Michaelis-Menton kintetics. Thus at substrate concentrations which are similar to or less than the K_m, the rate of the reaction is proportional to the substrate concentration. For simplicity, the CDP concentration was maintained in a steady state. This is the first model in which the law of mass action plays a role in regulating ribonucleotide reduction.

Jackson used this model to predict deoxyribonucleotide pool sizes and cell growth rates, in cultured N1S1 cells, following drug treatment. The qualitative agreement of the model with the data from intact cells was excellent but the quantitative agreement was not good. In two of the tests the model was qualitatively incorrect and the author pointed out that this could be a result of errors either in the rate equation itself, or in the kinetic parameters of the reductase as determined by Moore and Hurlbert.

Summary and Conclusions

Models 1 to 7 can be described as "first-generation" models in that they are based mainly on enzymological studies of ribonucleotide reductase. Models 8 to 10 can be termed "second-generation" models in that they are based to varying degrees on the "first-generation" models, and as well on the results of studies in intact cells. Finally, there are other models which are not discussed here, but which are best described as "illegitimate" in that they claim to be derived directly from a "first-generation" model, but in fact are misrepresentations of these models.

In order to compare the differences and similarities among the ten models described above more easily, Tables 10 to 13 consider each of the four ribonucleotide reduction reactions separately. As these Tables illustrate, the models often disagree on the effects of a given nucleotide effector on a particular reaction; however there are exceptions. For example, with one exception, all the models agree that dCTP has no effect on the reduction of any of the substrates. As well, ATP is not an inhibitior of any of the reactions, while dATP is always an inhibitor of ADP and GDP reduction and usually an inhibitor of CDP and UDP reduction as well. In the majority of the models, dTTP is an inhibitor of CDP and UDP reduction and a stimulator of GDP reduction, but never an inhibitor of ADP reduction.

Table 10

Effects of nucleotides on CDP Reduction

Model	Nucleotide				
	dTTP	dCTP	dGTP	dATP	ATP
1	I	O	O	I	S^R
2	I (ATP$^+$) S (ATP$^-$)	O	I (ATP$^+$) S (ATP$^-$)	I	S^r
3					
4	S	N.D.	S	S	N.D.
5	I	O	O	I	S
6	I	O	I	I	S
7	I	O	I	I	S
8	O	I	O	O	O
9	I	O	I	I	O
10	I	O	I	I	O

N.D. - not determined

Table 11

Effects of Nucleotides on UDP Reduction

Model	Nucleotide				
	dTTP	dCTP	dGTP	dATP	ATP
1	I	O	I	I	S
2	S(ATP$^-$) I(ATP$^+$)	O	O	I	S
3					
4	S	N.D.	O	S	N.D.
5	I	O	O	I	S
6	I	O	I	I	S
7	I	O	I	I	S
8	I	O	O	O	O
9	I	O	I	I	O
10	*	*	*	*	*

*UDP not included in model.

Table 12

Effects of Nucleotides on GDP Reduction

Model	Nucleotide				
	dTTP	dCTP	dGTP	dATP	ATP
1	S	O	I	I	S
2	S	O	S	I	O
3					
4	S	N.D.	S	I	N.D.
5	S	O	O	I	S
6	S	O	I	I	S
7	S	O	I	I	S
8	*	*	*	*	*
9	S	O	I	I	O
10	S/I[1]	O	I	I	O

*Not included in model.

[1] dTTP was defined as an activator at low concentrations and an inhibitor at high ones.

Table 13

Effects of Nucleotides on ADP Reduction

Model	Nucleotide				
	dTTP	dCTP	dGTP	dATP	ATP
1	S	O	S	I	O
2	S	O	S	I	O
3					
4	S	N.D.	S	I	N.D.
5	O	O	S	I	S
6	O	O	S	I	S
7	O	O	S	I	S
8	O	O	O	I	O
9	O	O	S	I	O
10	S	O	S	I	O

Finally, dGTP is almost always a stimulator of ADP reduction. Nevertheless, the amount of disagreement among these models demonstrates the need for further study. Aside from settling the above disagreements there are other enzymological questions which need to be answered, such as: "what is the effect of varying the concentration of one effector when all the known effectors are present at physiological concentrations?" Previous enzymological studies have only dealt with the effects of the presence of two effectors, at most.

EVALUATION IN INTACT MAMMALIAN CELLS

Four types of evidence have been adduced to support the idea that one or another enzymological model of ribonucleotide reductase regulation is indeed applicable to deoxyribonucleotide synthesis in intact mammalian cells. These are the following:

1) The toxicity of purine or pyrimidine deoxyribonucleosides. The toxicity of thymidine, deoxyadenosine, deoxyguanosine, etc. often are "explained" on the basis of models of ribonucleotide reductase regulation. Thus if the added deoxyribonucleoside is converted to the triphosphate, if the concentration of the triphosphate is elevated, if the additional triphosphate inhibits ribonucleotide reductase in the cell, if the synthesis of another deoxyribonucleoside triphosphate is blocked as a result, and if the

concentration of this triphosphate becomes limiting for DNA synthesis (or if DNA synthesis is inhibited due to an imbalance in concentrations of different triphosphates), then one could conclude that such toxicity provides evidence for the models described above.

At least in the case of purine deoxyribonucleoside toxicity (review: 15), the full chain of events has not been demonstrated, and in lieu of this all one can conclude is that deoxyribonucleoside toxicity is consistent with these models (or at least potentially consistent because of differences among them). At the present time observations of toxicity by themselves constitutes no proof of the models whatsoever.

2) Protection against deoxyribonucleoside toxicity by deoxycytidine. In many cases, purine and pyrimidine deoxyribonucleoside toxicity can be prevented by simultaneous addition of deoxycytidine and sometimes of other deoxyribonucleosides as well (alone or in combinations) (review: 15). Again, if the toxicity is in the first place in accord with one or another model of ribonucleotide reductase regulation, as described above, then concentrations of dCTP may fall and this may lead to inhibition of DNA synthesis. The effect of the added deoxycytidine, if it replenishes dCTP pools and thereby circumvents the inhibition of ribonucleotide reductase, would be in agreement with some or all of the models described above.

Again, however, this whole chain of events has not been demonstrated, and alternative explanations are possible. All one can conclude is that deoxycytidine protection is consistent with the models. At the present time, observations of deoxycytidine protection by themselves constitute no proof of the models.

3) Studies of the synthesis of radioactive deoxyribonucleotides from labeled ribonucleotides. To elevate the concentrations of individual deoxyribonucleoside triphosphates in a controlled and quantifiable fashion, and then to study the rates of reduction of all four radioactive ribonucleoside diphosphates in intact cells would provide the most direct and clear cut evidence for or against any of the models of ribonucleotide reductase regulation. Unfortunately, this approach is technically difficult to do well, and it has been attempted only in a few cases (16, review: 15); the results so far have not been conclusive.

4) Studies of the concentrations of deoxyribonucleoside triphosphates. Relatively recent improvements in the methodology of deoxyribonucleotide measurements have led to a number of studies in which the concentration of one deoxyribonucleoside triphosphate has been altered (usually increased) by one or another technique, the concentrations of one or all of the other triphosphates have been measured, and the results obtained have been compared with one or another of the models of ribonucleotide reductase regula-

tion described above.

Complications and Assumptions

Though some useful data has been gathered in studies such as those mentioned above, a number of real or potential complications often have not been taken into account.

1) The differences among the various enzymological models of ribonucleotide reductase regulation usually have neither been recognized, nor the consequences of having multiple models found.

2) In order for these data to be used to test the model, the data themselves should be evaluated using the following criteria:

a) All the deoxyribonucleoside triphosphate pools should be actually measured. Often it is assumed that, for example, addition of AdR or GdR to cells results in an increase in dATP or dGTP, respectively (16). This is not true in CHO-K1 cells where AdR and GdR are readily converted to ATP and GTP respectively (D. Hunting and J.F. Henderson, unpublished).

b) All the ribonucleoside triphosphate pools should be measured. ATP plays an important role in the ribonucleotide reductase models developed by Reichard by regulating both the general activity of the enzyme and the substrate specificity. Therefore, any test of the model must consider the possible effects of changes in ATP. As well, the possibility that changes in the other ribonucleoside triphosphate pools affect the deoxyribonucleotide pools should be considered.

c) In long term experiments under growth inhibitory conditions the cell cycle distribution of the population should be measured because shifts may occur, and cells in different phases of the cell cycle have different deoxyribonucleotide pool sizes (17; J.F. Henderson, unpublished).

3) As mentioned above, the most commonly used approach to the evaluation of models of the regulation of ribonucleotide reduction using intact cells is to measure concentrations of the deoxyribonucleoside triphosphates when the pool size of one or another of these is deliberately perturbed. This approach, however, really involves an important underlying assumption, namely that deoxyribonucleoside triphosphate concentrations reflect the rates of ribonucleotide reduction and this process only. In order for this to be true, three conditions must be met.

a) The rates of deoxyribonucleoside diphosphate phosphorylation must be equal to or greater than the rates of ribonucleotide reduction.

b) Deoxyribonucleotide catabolism must not compensate for changes in the rate of ribonucleotide reduction.

c) Deoxyribonucleotide consumption, via DNA synthesis or via catabolism, must be constant and must be sufficient to cause a triphosphate pool to decline if its synthesis is inhibited. Obviously if the rate of deoxyribonucleotide consumption responded immediately to decreases in the deoxyribonucleotide pools then large drops in pool size would not be observed.

These basic assumptions have not been tested directly, though there is some evidence available that pertains to each. Assumption a) seems reasonable in view of the fact that the deoxyribonucleoside diphosphate concentrations are a fraction of the triphosphate concentrations in cells, indicating that the equilibria favour the triphosphates. Furthermore, radioactive precursors such as [^{3}H]TdR and [^{3}H]CdR are readily phosphorylated to the triphosphate level, with less than 10% of the label being present as diphosphates.

We were unable to find published evidence relating to assumption b), and further study is required. We have found, using pulses of [^{3}H]CR and [^{3}H]UR, that deoxyribonucleosides do not accumulate in cells or in the medium either under control conditions or when the cells are pretreated with thymidine to perturb the deoxyribonucleotide pools.

There is good evidence that assumption c) is justified. Bjursell and Reichard have found that although the rate of DNA synthesis in S-phase CHO cells declined as the size of the dCTP pool declined during thymidine treatment, DNA synthesis continued even when the dCTP pool had decreased by 87% (7). Also, CHO cells continue to multiply, and therefore to synthesize DNA, even when the dCTP pool is 3% of control values (D. Hunting and J.F. Henderson, unpublished).

The Experimental Evidence

With these caveats in mind, it is now possible to review and evaluate critically the available experimental evidence that relates measurements of deoxyribonucleoside triphosphate concentrations to models of ribonucleotide reductase regulation. In the following discussion, the model of Thelander and Reichard (9) has been taken as the basis of comparison.

Lowe and Grindey (18) found that in L1210 cells treated with thymidine an increase in dTTP corresponded to a decrease in dCTP, but the dCTP concentration leveled off at ca. 70% of control values and remained constant up to the maximum value for dTTP achieved (500% of control). They found that a dGTP concentration of 150% of control corresponded to a dTTP concentration of 300% of control. Further increases in dTTP, up to 500% of control, had no further effect on dGTP. Although these results appear to agree with the model, it should be noted that these experiments were long-term, under growth inhibitory conditions, and ribonucleoside triphosphate concentrations were not measured.

Grindey _et_ _al_. (19) obtained similar results with CCRF-CEM cells treated with thymidine (21). They found that a dTTP concentration of 800% of control corresponded to a dCTP concentration of 58% of control, a dGTP concentration of 500% of control, a dATP concentration of 380% of control and an ATP concentration of 245% of control.

Unfortunately the increases in ATP and dATP make interpretation of the results difficult because Reichard's more recent ribonucleotide reductase models state that it is the ratio of ATP/dATP that determines the overall activity of the enzyme, but as well, an increase in ATP is predicted to increase pyrimidine reduction (6,9). Pool size measurement in these experiments were made after 45 hours under growth inhibitory conditions.

Tattersall *et al*. (20) using PHA-stimulated human lymphocytes treated with thymidine, found that a dTTP concentration of 600% of control corresponded to a dCTP concentration of 65% of control, a dGTP concentration of 118% of control and a dATP concentration of 0% of control. These results do not agree well with the model. The drastic decrease in the dATP concentration in particular is not explained by the model. With deoxyadenosine treatment they found that a concentration of dATP of 570% of control corresponded to a dTTP concentration of 22% of control and dCTP and dGTP concentrations of 0% of control. These results agree with the model. Treatment with deoxycytidine increased the dCTP pool to 890% of control and had no effect on the other deoxyribonucleoside triphosphate pools. Again, these results agree with the model which assigns no regulatory role to dCTP. The treatment time in these experiments was one hour, therefore significant cell synchronization could not have occurred; however, ribonucleotide concentrations were not measured.

Lowe et al. (21), using L5178Y cells treated with deoxyadenosine plus deoxycoformycin, found that dATP concentrations of 400% of control corresponded to dCTP concentrations of 45% of control and dGTP and dCTP concentrations of 100% of control. Ribonucleoside triphosphate pools were unchanged. These results do not agree with the model which predicts an increase in dATP to inhibit reduction of all substrates. These data were obtained using short-term incubations under growth inhibitory conditions. The cell cycle distribution, as measured by flow cytofluorometry, had not changed significantly.

Conclusions

Some experimental results appear to be consistent with the predictions of one or another model of the regulation of ribonucleotide reductase based on enzymological studies. However, not all of the data fit these models, and the basis and significance for these deviations are not yet known. The extent to which any of the models accurately portray the actual mode of regulation of ribonucleotide reductase in intact cells has yet to be determined.

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CHAPTER 8

EFFECTS OF ALTERED RIBONUCLEOTIDE CONCENTRATIONS ON RIBONUCLEOTIDE REDUCTION IN INTACT CHINESE HAMSTER OVARY CELLS

INTRODUCTION

The regulation of the enzyme ribonucleotide reductase (ribonucleoside diphosphate reductase, E.C. 1.17.4.1) and the regulation of ribonucleotide reduction in intact cells has been thought of mainly in terms of allosteric inhibition or stimulation by deoxynucleoside triphosphates (reviews: 1, 2). Several lines of evidence, however, indicate that ribonucleotide concentrations may also influence the rate of ribonucleotide reduction, whether in intact cells or purified enzyme preparations.

First, a number of enzymological studies have shown that ATP stimulates ribonucleotide reductase activity, mostly with respect to the reduction of CDP and UDP. Thus, Moore and Hurlbert found that reduction of CDP, UDP and GDP was stimulated by ATP (3), while Larsson and Reichard observed stimulation of CDP and UDP reduction by ATP (4). The stimulation of pyrimidine reduction by ATP was also included in the models of the regulation of this enzyme proposed by Reichard (review: 1) and by Thelander and Reichard (5). The only other ribonucleotide which, based on enzymological studies, affects ribonucleotide reduction is UTP; in one study (4) it was found to stimulate the reduction of GDP.

Studies of ribonucleotide reductase have also shown that whereas dATP was an inhibitor of the reduction of all four substrates, this effect could be overcome by ATP.

Reichard has therefore proposed that the enzyme contains an allosteric "activity" site that binds either ATP or dATP (1).

Another type of evidence for the role of substrates in the regulation of ribonucleotide reduction is provided by competition experiments between substrates. Thus Larsson and Reichard, using purified E. coli reductase, found that UDP was a competitive inhibitor of CDP reduction and vice-versa, while GDP was a competitive inhibitor of CDP reduction (4). As well, Eriksson et al. using purified calf thymus reductase (6), and von Döblen and Reichard using purified E. coli reductase (7), found that CDP and GDP competed with each other for the same catalytic site. Therefore, even in the absence of other effects, one might expect that an increase in the concentration of one substrate could increase its rate of reduction while inhibiting the reduction of other nucleotides.

In addition, the apparent Michaelis constants for the four ribonucleoside diphosphate substrates are in the range of intracellular concentrations of these nucleotides. Thus K_m's of 20 to 80 μM and 40 to 100 μM have been reported for enzyme preparations from Novikoff hepatoma (3), E. coli (4), and calf thymus (6), respectively, while values for ribonucleoside diphosphate concentrations have been reported to vary from 30 to 1700 μM (review: 8). It is therefore possible that mass action plays a role in determining the rate of ribonucleotide reduction in intact cells, especially if nucleotide concentrations are altered by drug treatment.

Studies of some drugs that lower concentrations of certain ribonucleotides (methotrexate (9), mycophenolic acid (10), 6-methylmercaptopurine ribonucleoside (11)), showed that concentrations of the corresponding deoxyribonucleotide(s) were also reduced.

Finally, while studying the allosteric regulation of ribonucleoside reduction in intact cells, we noted that some deoxyribonucleotide triphosphate pool size changes did not correlate with changes in other deoxyribonucleoside triphosphate pools, but did seem to correlate with changes in the corresponding ribonucleoside triphosphate. This prompted further investigations to determine the effect of changes in each ribonucleoside triphosphate concentration on the concentration of the corresponding deoxyribonucleoside triphosphate. The results of this study are presented here.

MATERIALS AND METHODS

[^{3}H]dATP, 11 Ci/mmol, [^{3}H]dTTP, 14 Ci/mmol, [^{3}H]dCTP, 16.8 Ci/mmol, and [^{3}H]dGTP, 15 Ci/mmol, were obtained from ICN Pharmaceuticals, and [8-^{3}H]adenosine, 24 Ci/mmol, from Amersham-Searle. Poly[d(IC)] was purchased from Miles Laboratories, poly[d(AT)], and non-radioactive purine and pyrimidine bases, nucleosides, nucleotides and 6-azauridine, from Sigma Chemical Co. Mycophenolic acid, pyrazofurin, 3-deazauridine and PALA (N-phosphonoacetyl-L-aspartate) were gifts of the U.S. National Cancer Institute.

The experimental approach used was to manipulate intracellular concentrations of ribonucleotides using drugs of high specificity. Conditions were chosen, by trial and error, such that no significant changes in cell volume or cell cycle distribution occurred. Changes in ribonucleoside triphosphate concentration were plotted against the corresponding deoxyribonucleotide concentration. In order to determine the temporal relationship of the pool size changes, changes in ribo- and deoxyribonucleoside triphosphate concentrations were plotted against time.

Cell culture. Chinese hamster ovary-K1 cells, obtained from Dr. G. Whitmore (Ontario Cancer Institute, Toronto, Ontario), were grown in alpha-MEM medium containing 10% dialyzed fetal calf serum (Grand Island Biological Co.). The cells were grown in 125 ml bottles on a Model G-2 gyrorotary shaker (New Brunswick Scientific Co.) at 200 rpm. The average doubling time was 12 h and the growth rate was exponential to 0.8-1.0 x 10^6 cells/ml. The cells were routinely tested for mycoplasma by the Department of Medical Bacteriology, University of Alberta, Edmonton, Alberta, and found to be negative.

Biological parameters. Cell density and population volume distribution were determined using a Model Z_F Coulter Counter equipped with a 100-channel Coulter Channelyzer II. The cell cycle distribution was determined by flow microfluorometry using a Bio/Physics Model 4800A Cytofluorograph equipped with a 100-channel Model 2100 pulse height analyzer. The cells were collected in a clinical centrifuge, resuspended to 400,000 cells/ml in 0.05 mg/ml propidium iodide in 0.1% sodium citrate, and stained for 20 to 40 min on ice. In any given experiment, the staining time was identical for all the samples.

Cell extraction. Preparation of extracts for nucleotide pool size measurement was as follows: 0.25-4.0 x 10^7 cells were centrifuged at 1000 g for 2 min at 4°C. The medium was aspirated and the tube recentrifuged at 1000 g for 5 sec to remove medium from the centrifuge tube wall. The pellet was extracted on ice with 0.4 M PCA containing [^{3}H]adenosine for determination of dilution. After 30 min the extract was centrifuged and the supernatant was removed and neutralized by extraction with 0.5 M Alamine 336 (tricapryl tertiary amine) in Freon-TF (trichlorotrifluoroethane) (12). Supernatants were stored at -20°C. Analysis by HPLC of samples stored for several weeks showed no nucleotide breakdown.

Ribonucleotide concentrations. Ribonucleotide concentrations were measured by HPLC using a modified Varian Aerograph 1000 liquid chromatograph equipped with a Spectra-Physics Model 740P pump, a Waters Associates U6K injector, and a Spectra-Physics Autolab Minigrator. A Partisil 10 SAX anion exchange column (Whatman) was used, and the nucleotides were routinely eluted isocratically at 38°C with 0.25 M KH_2PO_4, 0.5 M KCl, pH 4.5 at 1.3 ml/min. This allowed quantitation of ADP and the triphosphates. When values for the mono-, di- and triphosphates were required, a linear gradient from 0.015 M KH_2PO_4, pH 4.0 to 0.5 M KH_2PO_4, 1.0 M KCl, pH 4.5 was used. Detection was at 254 nm and at 0.02 absorbance units full scale. The peaks were automatically integrated and also checked by planimetry. The absolute amounts of nucleotides in the sample were calculated on the basis of peak areas of nucleotide standards which were chromatographed frequently.

Deoxyribonucleoside triphosphate concentrations. The following components were common to both the dATP and dTTP assay in a final volume of 180 µl: 0.02 A_{260} units poly-[d(AT)], 1.8 µmoles $MgCl_2$, 1.8 µmoles dAMP, 18 µmoles Hepes buffer, pH 7.4, and 0.75 Richardson units of DNA polymerase I (13). As well, the dATP assay contained 100 pmoles (0.5 µCi) $[^3H]$dTTP, and 0 to 75 pmoles dATP standard, while the dTTP assay contained 100 pmoles (0.5 μCi) [^{3}H]dATP and 0 to 75 pmoles dTTP standard.

The following components were common to both the dGTP and the dCTP assays in a final volume of 180 μl: 0.02 A_{260} units poly[d(IC)], 1.8 μmoles $MgCl_2$, 1.8 μmoles dAMP, and 18 μmoles Hepes buffer, pH 7.4. As well, the dGTP assay contianed 100 pmoles (2.2 μCi) [^{3}H]dCTP, 0 to 10 pmoles dGTP standard and 1.9 units DNA polymerase I. The dCTP assay contained 240 pmoles (0.5 μCi) [^{3}H]dGTP, 0 to 200 pmoles dCTP standard and 3.0 units DNA polymerase I.

The reaction was started by the addition of the DNA polymerase I, followed by incubation at 37°C. At each time point aliquots were removed and spotted on squares of Whatman 3MM filter paper which had been wetted with 200 μl of 2% sodium pyrophosphate. The squares were washed (3 x 15 min) with a solution of 5% TCA and 1% sodium pyrophosphate (20 ml/square), then rinsed once with 95% ethanol and finally washed (1 x 15 min) with 95% ethanol. The dried filters were counted in toluene scintillation solution containing 4 g PPO and 0.1 g POPOP per liter toluene.

Results were corrected for the washing background and for the effects of the dilution of the specific activity of the labeled deoxyribonucleotide by the sample on both the sample incorporation and the background incorporation.

Purity of reagents. The purity of the unlabeled deoxyribonucleoside triphosphates was 90-98 molar % as determined by HPLC. The impurities were deoxyribonucleoside mono- and diphosphates, which have the same extinction coefficients as the triphosphates. The concentrations of the standard solutions were determined by measuring the uv absorbance and correcting for the presence of mono- and diphosphates. The standard nucleotide solutions were stable for several months at -20°C.

DNA polymerase I was supplied and stored in 50% glycerol, pH 7.0. A working solution was prepared by diluting the stock solution with 50 mM Tris-HCl, pH 7.8 containing 12 mg/ml bovine serum albumin. This solution was stored not longer than 1 month.

The (*E. coli*) DNA polymerase purchased from Boehringer Mannheim Corp. was not contaminated with phosphatase activity, but samples obtained from other sources have been. Each new batch of enzyme was checked for phosphatase activity.

The following controls were performed: background incorporation (i.e., in the absence of the limiting non-radioactive deoxyribonucleotide) was always measured. In addition, standards were added to cell extracts to determine if the assay was affected by the extracts, and checks were made to determine that the assays were independent of the amount of extract used. Finally, time courses were always performed both with standards and with each cell extract to ensure that the maximum incorporation was reached at the same time under all conditions.

Manipulation of ribonucleotide concentrations. Cells were incubated with naturally occurring bases and nucleosides or with drugs in order to raise and lower ribonucleotide concentrations. Bases and nucleosides used included cytidine, uridine, deoxycytidine, thymidine, adenine and guanosine. Drugs used included mycophenolic acid, which inhibits inosinate dehydrogenase (14,15); pyrazofurin, and 6-azauridine, which inhibit orotidylate decarboxylase (16,17): PALA, which inhibits aspartate transcarbamylase (18); 3-deazauridine, which inhibits CTP synthetase (19); and tetrahydrouridine, which inhibits cytidine-deoxycytidine deaminase (20). In several instances, secondary effects of the drugs were made use of in order to manipulate ribonucleotide pools.

CTP concentrations were lowered by incubating cells with 2, 5 and 10 μM 3-deazauridine for 2, 4 and 6 h; with 0.1 μM 6-azauridine for 6 h; with 500 μM PALA for 2, 4 and 6 h; or with 0.01 and 0.2 μM pyrazofurin for 2, 4 and 6 h. CTP concentrations were elevated using 2.0 μM mycophenolic acid for 2 to 6 h; 600 μM cytidine for 20, 40, 60, 80 and 100 min; 1000 μM cytidine for 12 h; 1000 μM cytidine plus 100 μM tetrahydrouridine for 12 and 21 h; or 2.0 μM mycophenolic acid plus 0.1 μM 6-azauridine for 6 h.

UTP concentrations were lowered by incubating cells with 0.01 and 0.2 μM pyrazofurin for 2, 4 and 6 h; with 500 μM PALA for 2, 4 and 6 h; with 0.1 μM 6-azauridine for 4, 6 and 18 hours; with 1000 μM deoxycytidine plus 100 μM tetrahydrouridine for 12 h; and with 1000 μM cytidine plus 100 μM tetrohydrouridine for 12 h. UTP concentrations were elevated when cells were incubated with 900 μM uridine for 20, 40, 60, 80 and 100 min; 0.5 or 2.0 μM mycophenolic acid for 2 to 6 h; and with 2.0 μM mycophenolic acid plus 0.1 μM 6-azauridine for 6 h.

ATP concentrations were lowered in the presence of 1000 μM cytidine for 12 h; 1000 μM cytidine plus 100 μM tetrahydrouridine for 12 h; 1000 μM deoxycytidine plus 100 μM tetrahydrouridine for 12 h; 2, 5 or 10 μM 3-deazauridine for 4 h; and of 100 or 2000 μM deoxythymidine for 4 and 6 h. ATP concentrations were elevated by treatment with 200 μM adenine for 20, 40, 60, 80 and 100 min; 0.01 μM pyrazofurin for 4 and 6 h; 0.1 μM 6-azauridine for 4 and 18 h; and with 2.0 μM mycophenolic acid plus 0.01 μM pyrazofurin for 6 h.

GTP concentrations were lowered by treating cells with 0.5 and 2.0 μM mycophenolic acid for 2, 4 and 6 h; 2.0 μM mycophenolic acid plus 0.01 μM pyrazofurin for 6 h; with 2.0 μM mycophenolic acid plus 0.1 μM 6-azauridine for 6 h; with 2.0 μM 3-deazauridine for 4 and 6 h; and with 1000 μM cytidine for 12 h. GTP concentrations were elevated by incubation with 500 μM guanosine for 20, 40, 60, 80 and 100 min.

RESULTS

The relationship between ribonucleotide concentrations and rates of reduction of ribonucleoside diphosphates to deoxyribonucleotides was assessed by measuring deoxyribonucleoside triphosphate concentrations in cells that contained a range of concentrations of each ribonucleoside triphosphate. (Triphosphate concentrations are reported in each case because they are more easily and more accurately measured than the diphosphates and because concentrations of ribonucleoside diphosphates were consistently ca. 10% of those of the triphosphates. Likewise, the phosphorylation of the deoxyribonucleoside diphosphates to the triphosphates seems to be rapid; this is true also of the conversion of dUDP to dTTP.)

To raise or lower ribonucleotide pools, Chinese hamster ovary cells were incubated with appropriate drugs or substrates; the conditions chosen were based on a broad survey of conditions that will alter nucleotide concentrations. The data reported here were obtained either under conditions that caused no growth inhibition, or using short-term incubations (2-6 h) under conditions which at 24 h were growth inhibitory; in the latter cases the conditions used caused no alterations in mean cell volumes or in cell cycle distribution.

Results are presented both to show correlations between ribo- and deoxyribonucleotide concentrations, and to show time courses of changes in each type of pool. Data are expressed as percent of control values, and in the correlation plots only ribonucleoside triphosphate concentrations that had changed 20% or more are plotted; therefore, control values are not included in these plots.

CTP and dCTP Concentrations

Figure 1 shows the relationship between CTP and dCTP over a wide range of CTP concentrations. These data show that dCTP concentrations reflected those of CTP, whether the latter had been increased or decreased relative to control values. The probability that the correlation coefficient was zero was less than 1×10^{-7}, as determined using a t-test on the correlation coefficient.

Using a different type of data presentation, given in Figure 2, CTP concentrations are shown to decrease with time in cells treated with PALA (Figure 2a), pyrazofurin (Figure 2b) and 3-deazauridine (Figure 2c), and to decrease and increase in cells treated with mycophenolic acid (Figure 2d). The concentrations of dCTP in each case change at almost the same rate, and to almost the same extent, as do those of CTP. The basis for the biphasic changes in nucleotide concentrations in cells treated with mycophenolic acid is not known. (Though growth inhibition was demonstrated at the longer times shown here, the major changes in nucleotide concentrations occurred before any changes on cell cycle or growth parameters were noticeable.)

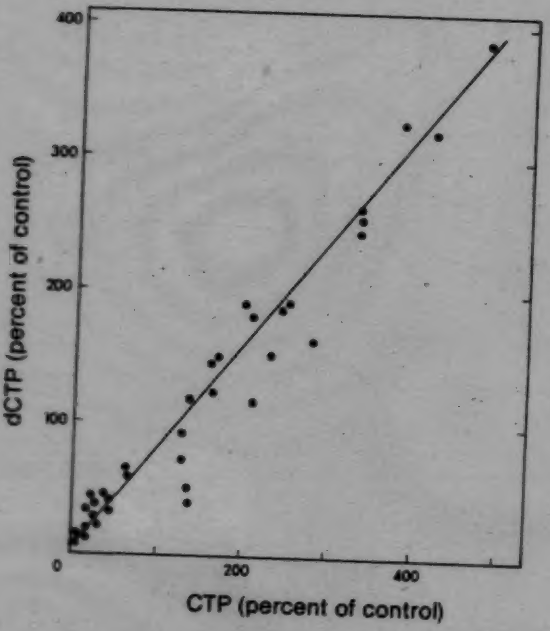

FIG. 1. Effect of changes in the concentrations of CTP on those of dCTP. Control values (pmol/10^6 cells): CTP, 1230; dCTP, 223.

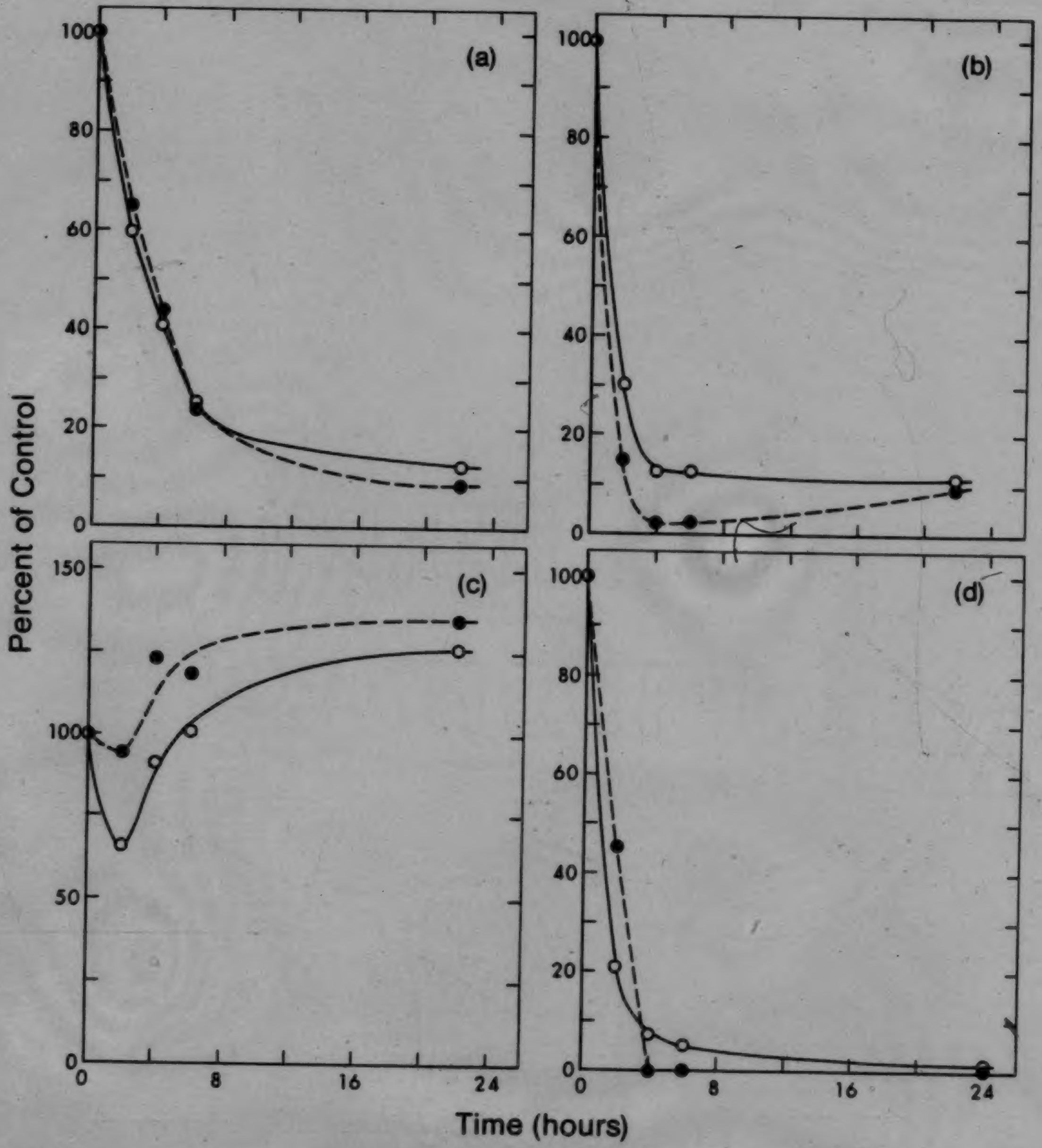

FIG. 2. Changes in the concentrations of CTP (●) and dCTP (○) during treatment with (a) PALA, (b) pyrazofurin, (c) 3-deazauridine and (d) mycophenolic acid.

UTP and dTTP Concentrations

Figure 3 shows the relationship between UTP and dTTP over a wide range of UTP concentrations. These data show that dTTP concentrations reflected those of UTP, though the relationship was not as direct or simple as with CTP and dCTP. Nevertheless, both increases and decreases in UTP led to corresponding changes in dTTP. The probability that there is no correlation is less than 1×10^{-7}.

Figures 4a and 4b show that when cells were treated with either PALA or pyrazofurin, UTP concentrations decreased rapidly, though pyrazofurin was slightly more effective than PALA. Though dTTP concentrations also declined, this occurred more slowly and to a lesser extent, than did UTP. Figure 4c shows changes in UTP and dTTP during treatment of cells with mycophenolic acid. UTP reached a maximum value of 145% of control within 4 h, whereas dTTP continued to increase as long as 24 h; dGTP concentrations reached their minimum value after 4 h of treatment.

ATP and dATP Concentrations

The data in Figure 5 demonstrate a significant correlation between changes in ATP concentration and those of dATP, though there was more scatter in the data and the range of ATP concentrations attained was less than those used for CTP and UTP; the probability that the correlation coefficient is zero is less than 1×10^{-4}.

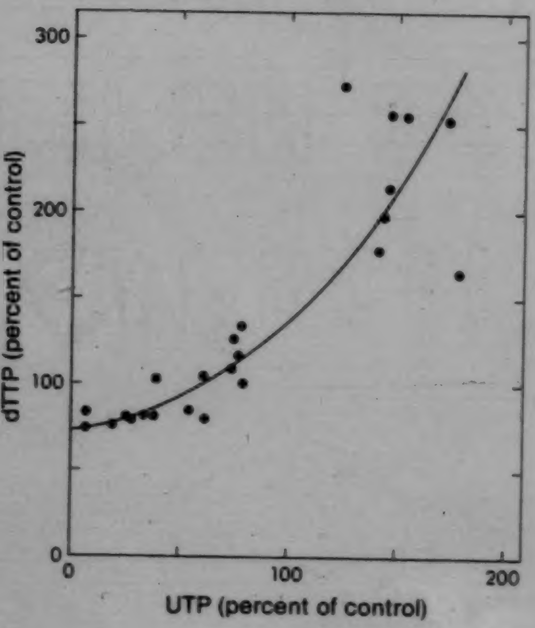

FIG. 3. Effect of changes in the concentration of UTP on those of dTTP. Control values (pmol/10^6 cells): UTP, 2390; dTTP, 58.

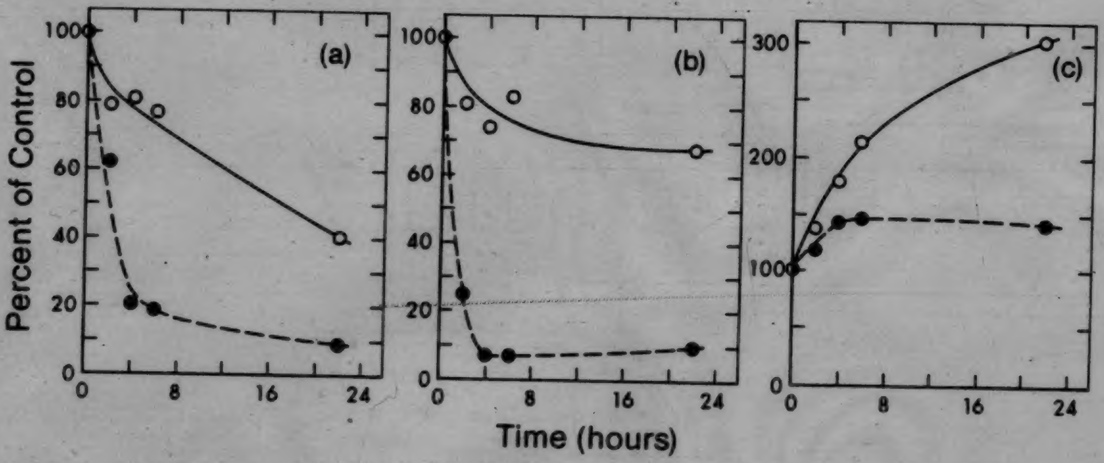

FIG. 4. Changes in the concentrations of UTP (●) and dTTP (○) during treatment with (a) PALA, (b) pyrazofurin, and (c) mycophenolic acid.

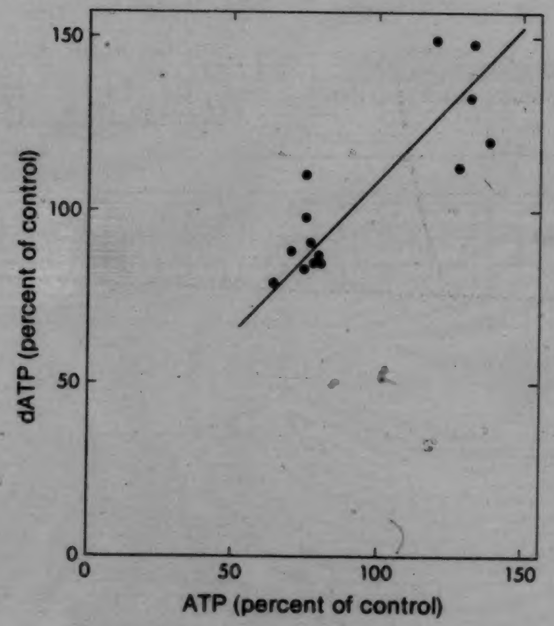

FIG. 5. Effect of changes in the concentrations of ATP on those of dATP. Control values (pmol/10^6 cells): ATP, 5580; dATP, 39.

GTP and dGTP Concentrations

The relationship between concentrations of GTP and those of dGTP is shown in Figure 6. Again, both increases and decreases in GTP were associated with corresponding changes in dGTP concentrations. Figure 7 shows that GTP concentrations fell rapidly in cells treated with mycophenolic acid, and that those of dGTP decreased to the same relative extent and at the same rate.

DISCUSSION

The question of the relationship between ribonucleotide concentrations and those of the corresponding deoxyribonucleoside triphosphates, may be thought of in terms of two related but distinct questions: (a) do deoxyribonucleoside concentrations increase when that of the corresponding ribonucleotide is elevated? (b) do deoxyribonucleoside triphosphate concentrations decrease when that of the corresponding ribonucleotide is lowered?

Basically, the results obtained in this study answer both questions in the affirmative. However, some differences were noted among the four "families" of nucleotides. The most linear relationship over a wide range of concentrations was obtained with CTP and dCTP. The relationship between GTP and dGTP concentrations was also relatively direct, though the range of GTP concentration was narrower. Only limited data were available for ATP and dATP, and in fact the data appear to fall in two clusters. The most

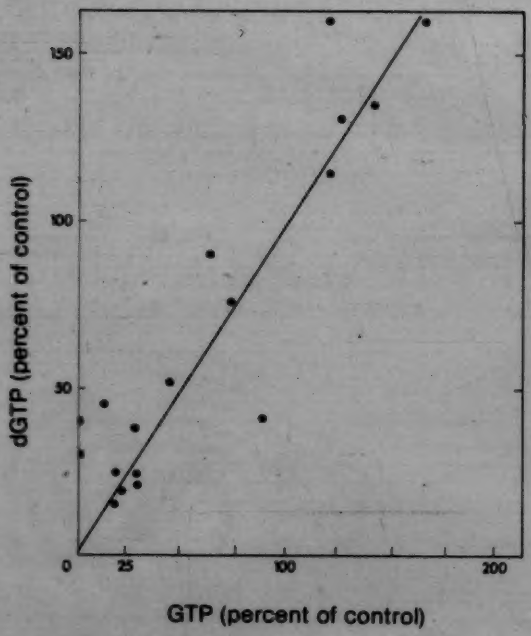

FIG. 6. Effect of changes in the concentrations of GTP on those of dGTP. Control values (pmol/10^6 cells): GTP, 1070; dGTP, 12.

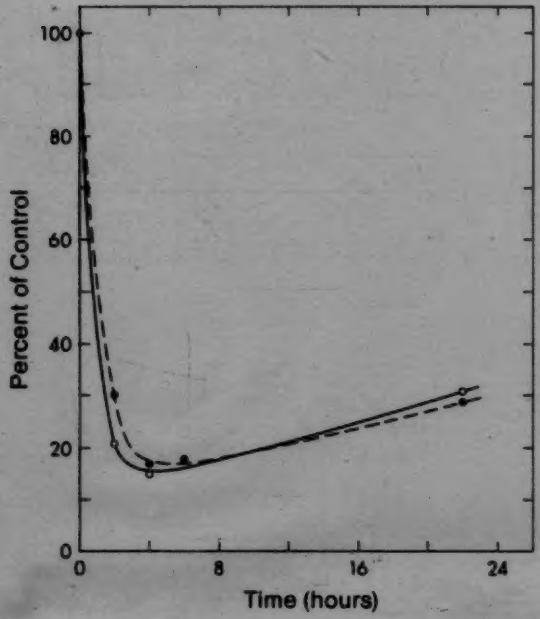

FIG. 7. Changes in the concentrations of GTP (●) and dGTP (○) during treatment with mycophenolic acid.

interesting results were obtained with UTP and dTTP, in which case changes in dTTP were not as great nor as rapid as those in UTP. Whether this is because dCMP deaminase plays an important role in thymidylate synthesis, or for some other reason, remains to be investigated further.

The mechanism or mechanisms by which changes in ribonucleotide concentrations led to changes in those of the deoxyribonucleoside triphosphates has not been studied. The possibility must be considered that the considerable variety in experimental conditions used to raise and lower ribonucleotide concentrations, especially through the use of drugs, may have had secondary consequences that obscured or distorted the results. Though this question must to a certain extent always remain open in this type of study, the fact that the results obtained generally were consistent from one set of conditions to another, tends to suggest that this was not a major problem.

The most simple explanation for these results is simply based on mass action; the rate of ribonucleotide reduction is dependent on the concentration of the substrates. This conclusion of course needs to be tested by more direct measurements of ribonucleotide reduction using radioactive precursors.

The principal alternative explanation that must be considered is that changes in the concentration of one deoxyribonucleoside triphosphate may lead to changes in that of another deoxyribonucleoside through allosteric inhibition or inhibition of ribonucleotide reductase. For example, according to the Thelander and Reichard model (5), CDP reduction should be inhibited by elevated dATP, dGTP or dTTP. At the lowest concentrations of dCTP achieved, using PALA, pyrazofurin or 3-deazauridine, the allosteric inhibitors of CDP reduction were generally not appreciably elevated. For example, with 3-deazauridine treatment, dTTP was the only allosteric effector that was elevated, and values of dCTP of 5 and 7% of control corresponded to dTTP concentrations of 111 and 123% of control respectively. With pyrazofurin treatment, dATP was the only allosteric effector that was elevated, and a dCTP concentration of 13% of control corresponded to dATP concentrations of 109 and 113% of control. None of the allosteric effectors were elevated with PALA treatment and in fact at a dCTP concentration of 25% of control, dGTP and dTTP were 51 and 77% of control. This is contrary to the allosteric model, because lowering the concentration of inhibitors should stimulate CDP reduction.

According to the Thelander and Reichard model, CDP reduction should be stimulated by a slight elevation of dATP, or by decreases in dGTP or dTTP. The highest concentrations of dCTP were achieved with cytidine and tetrahydrouridine plus cytidine. Under these conditions, dGTP was the only allosteric effector that was significantly lowered. For example, concentrations of dCTP of 190% and

264% of control corresponded to dGTP concentrations of 41% and 26%, respectively, and to CTP concentrations of 244% and 343% of control, respectively. In this case, it appears that the results are consistent with the allosteric model as well as with the law of mass-action; however, situations where dGTP is low (i.e. 42% of control) and dCTP is not elevated (as occurs during pyrazofurin treatment) argue against dominant control by the allosteric effectors under these conditions.

The data presented in Figure 5 can be lumped into two groups - slightly elevated dATP, achieved by treatment with adenine, pyrazofurin, 6-azauridine and mycophenolic acid plus pyrazofurin, and slightly lowered dATP, achieved by treatment with cytidine, cytidine plus tetrahydrouridine, deoxycytidine plus tetrahydrouridine, 3-deazauridine, and thymidine. The allosteric model predicts that elevated dATP could be caused by an elevation of dGTP. The treatments which result in elevated dATP actually lower dGTP significantly (79 to 41% of control) and therefore, this situation cannot be explained by the allosteric model, but can be explained by the law of mass action. The treatments which lower dATP have no consistent effect on dGTP, and in fact, dATP concentrations of 83% of control correspond to dGTP concentrations of 190% of control while ATP concentrations are 75% of control. In other cases, dATP concentrations of 85% of control correspond to dGTP concentrations of 85% of control and to dATP concentrations of 78% of control.

The allosteric model of Thelander and Reichard predicts that a decrease in dTTP or a large increase in dATP would result in a decrease in dGTP. In fact, the lowest concentration of dGTP (15 to 21% of control) corresponded to substantially elevated concentrations of dTTP (137 to 214% of control) and to dATP concentrations of 82 to 163% of control. None of the treatments which resulted in lowered dGTP, caused an elevation of dTTP, and the majority did not elevate dATP. Thus, under these conditions the allosteric model, as proposed by Thelander and Reichard, does not explain the changes in the dGTP concentration. Clearly, further studies need to be undertaken of the specific mechanism(s) by which changes in ribonucleotide concentrations influence the concentrations of the deoxyribonucleoside triphosphates.

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CHAPTER 9

REGULATION OF RIBONUCLEOTIDE REDUCTION BY DEOXYRIBONUCLEOTIDES IN INTACT CHINESE HAMSTER OVARY CELLS

INTRODUCTION

Ribonucleotide reductase (ribonucleoside diphosphate reductase, EC 1.17.4.1) is essential for the synthesis of the substrates for DNA synthesis in most mammalian cells. Numerous enzymological studies have shown that its activity is regulated by deoxyribonucleoside triphosphates and by some ribonucleotides, and a number of models of the action and regulation of this enzyme have been formulated.

One model for the *in vivo* regulation of ribonucleotide reductase from Novikoff hepatoma cells was published by Moore and Hurlbert in 1966 (1), and in the same year a model for the *in vivo* regulation of the *E. coli* enzyme was published by Larsson and Reichard (2,3). Superficially, the two models were similar, but there also were many important differences. In 1969, Brown and Reichard substantiated many of the features of the previous *E. coli* model by carrying out binding studies on highly purified ribonucleotide reductase (4). Up to 1978, these two models, especially the *E. coli* model, provided the major basis for thinking not only about the regulation of ribonucleotide reduction *in vivo*, but also about the control of DNA synthesis, and about mechanisms of toxicity of compounds such as deoxyadenosine, deoxyguanosine and deoxythymidine. Then in 1978, Reichard published a new model for the *E. coli* reductase which differed substantially from his earlier model (5). Subsequently, Eriksson, Thelander and Akerman published a model for the *in vivo* regulation of the reductase from calf thymus, which shared many of the features of Reichard's 1978 model but also showed significant differences (6). Finally Thelander and Reichard recently have published a model that appears to be a composite of the *E. coli* and the calf thymus reductase models (7). These authors state: "*In vitro*, the activity and substrate specificity of the reductase from *E. coli* and mammalian sources behave similar (sic) towards allosteric effectors. The data can be integrated into a scheme that links ribonucleotide reduction to DNA synthesis." Other models have also been described, based usually on one or another of the above-mentioned schemes, or on combinations of several models (e.g., 8-10).

Several studies that have measured deoxyribonucleoside triphosphate concentrations in mammalian cells grown or incubated under various conditions have obtained results which at least in part are in general accord with one or another model of ribonucleotide reductase (e.g., 11-15). In general these studies either were not specifically designed to test models of ribonucleotide reductase regulation, or were incomplete in some way.

In this study an attempt has been made to assess the extent to which the recent Thelander-Reichard model of ribonucleotide reductase regulation by deoxyribonucleoside triphosphates (7) applies to ribonucleotide reduction in cultured Chinese hamster ovary cells.

The results of the experiments indicate that: 1) portions of this model of ribonucleotide reductase regulation may apply in intact cells, 2) other components of this model have no relevance to CHO cells, and 3) the model is not sufficient to explain all deoxyribonucleotide pool size changes *in vivo*.

MATERIALS AND METHODS

Sources of materials, and methods for the culture of CHO-K1 cells in alpha MEM medium containing 10% dialyzed fetal calf serum, for the extraction of nucleotides, and the measurement of ribo- and deoxyribonucleotide concentrations, are described in the preceeding paper.

Manipulation of Deoxyribonucleotide Concentrations

Cells were incubated with naturally occurring nucleosides or with drugs in order to raise or lower deoxyribonucleotide concentrations. Nucleosides used included cytidine, deoxycytidine and thymidine. Drugs used included mycophenolic acid, which inhibits inosinate dehydrogenase; 6-azauridine and pyrazofurin, which inhibit orotidylate decarboxylase; PALA, which inhibits aspartate transcarbamylase; 3-deazauridine, which inhibits CTP synthetase; and tetrahydrouridine which inhibits cytidine-deoxycytidine deaminase. In several instances, secondary effects of the drugs were made use of in order to manipulate the deoxyribonucleotide pools.

The effect of changes in dTTP concentrations on dCTP concentrations was studied under the following conditions: 0.5 μM and 2.0 μM mycophenolic acid for 2 and 6 h; 2 mM deoxycytidine for 21 hr; 0.01 μM pyrazofurin plus 2 μM mycophenolic acid for 6 h; 0.01 μM 6-azauridine plus 2 μM mycophenolic acid for 6 h; and 0.1, 0.5 and 2.0 mM thymidine for 2, 4 and 6 h.

These conditions did not cause significant changes in CTP concentrations.

The effect of changes in dTTP concentrations on dGTP concentrations was studied under the following conditions: 0.01 and 0.02 μM pyrazofurin for 4 and 6 and 2, 4 and 6 h, respectively; 500 μM PALA for 2, 4 and 6 h; 1.0 mM deoxycytidine for 21 h; 1 mM deoxycytidine plus 0.1 mM tetrahydrouridine for 21 h; 2, 5 and 10 μM 3-deazauridine for 2, 4 and 6 h and 0.1, 0.5 and 2.0 mM thymidine for 2, 4 and 6 h.

These conditions did not cause significant changes in GTP concentrations. It was not possible to achieve very low concentration of dTTP using short-term incubations with pyrazofurin or PALA. Attempts to lower dTTP concentrations with 6-azauridine, to study its effect on dGTP concentrations, were abandoned when it was found to inhibit GTP synthesis.

The effect of changes in dGTP concentrations on dCTP concentrations was studied under the following conditions: 0.1, 0.5 and 2.0 mM thymidine for 2,4 and 6 h; 2 μM 3-deazauridine for 2 h; 0.1 mM tetrahydrouridine for 6, 10 and 24 h, 1 mM deoxycytidine plus 0.1 mM tetrahydrouridine for 12 h; and 1 mM deoxycytidine for 12 and 21 h. Deoxycytidine caused a substantial increase (>200%) in dGTP concentrations and lowered dCTP concentrations. This makes it seem unlikely that deoxycytidine was contributing significantly to the dCTP pool.

These conditions did not have a significant effect on CTP concentrations.

The effect of changes in dGTP concentrations on dTTP concentrations was studied under the following conditions: 0.5 and 2.0 μM mycophenolic acid for 6 and 2 hr, respectively; 1.0 mM cytidine for 12 h; 1.0 mM deoxycytidine plus 0.1 mM tetrahydrouridine for 12 and 21 h, 2.0 μM mycophenolic acid plus 0.01 μM pyrazofurin for 6 hr, and 2, 5 and 10 μM 3-deazauridine for 2, 4 and 6 h.

These conditions did not have a significant effect on UTP concentrations.

The effect of changes in dGTP concentrations on dATP concentrations was studied under the following conditions: 0.5 and 2 μM mycophenolic acid for 2, 3, 4 and 6 h; 500 μM PALA for 2, 4 and 6 h; 1.0 mM deoxycytidine for 21 h; 1.0 mM deoxycytidine plus 0.2 mM tetrahydrouridine for 12 and 21 h; 2.0 μM mycophenolic acid plus 0.01 μM 6-azauridine for 6 h; 2, 5 and 10 μM 3-deazauridine for 2, 4 and 6 h; and 0.1, 0.5 and 2.0 mM thymidine for 2, 4 and 6 h.

These conditions did not have a significant effect on ATP concentrations. It was not possible to increase dGTP concentrations using deoxyguanosine because it was rapidly phosphorolysed. Therefore, the highest concentrations of dGTP were obtained using thymidine.

The effect of changes in dATP concentrations on dCTP concentrations was studied under the following conditions: 0.5 and 2 μM mycophenolic acid for 2, 3, 3.5, 4 and 6 h; 1.0 mM deoxycytidine for 21 h; 1.0 mM deoxycytidine plus 0.1 mM tetrahydrouridine for 12 h; 2 μM mycophenolic acid plus 0.01 μM pyrazofurin for 6 h; 2 μM mycophenolic acid plus 0.01 μM 6-azauridine for 6 h; and 2 μM 3-deazauridine for 2 h.

These conditions did not have a significant effect on the CTP pool.

None of the conditions described above had a significant effect on cell volume, as determined on a Coulter Channelyzer, or on cell cycle distribution as determined by flow microfluorometry.

RESULTS

The experimental approach used to test the Thelander-Reichard model of the regulation of ribonucleotide reduction by deoxyribonucleoside triphosphates was to treat cultured Chinese hamster ovary cells with drugs or with naturally occurring deoxyribonucleosides in order to alter the concentration of a particular deoxyribonucleoside triphosphate. Concentrations of all ribo- and deoxyribonucleoside triphosphates were then measured. Data (expressed as percent of control values) are presented (a) to show the correlation between the concentrations of two deoxyribonucleoside triphosphates (usually without regard for the conditions through which concentrations were achieved), and (b) to show the time courses of nucleotide concentration changes under a few selected conditions.

Effect of dTTP on CDP Reduction

The Thelander-Reichard model of ribonucleotide reductase regulation predicts that an increase in dTTP concentration will inhibit the reduction of CDP. Therefore, as dCTP is consumed through incorporation into DNA, the concentration of dCTP will decrease.

The data in Fig. 1 show that there was an inverse relationship between dTTP and dCTP concentrations. At dTTP concentrations less than 600% of control values, dCTP concentration appeared to vary markedly with changes in dTTP, whereas at higher levels of dTTP, dCTP concentrations appeared to be much less sensitive to increases in dTTP. Even at very high dTTP concentrations (2700% of control), dCTP was still present at levels of 3% of control. The conditions used in these experiments caused no changes in the concentration of CTP.

In order to determine the temporal relationship between dTTP and dCTP pool size changes, cells were treated with several concentrations of thymidine in order to increase the dTTP pool. The results of treatment with 2.0

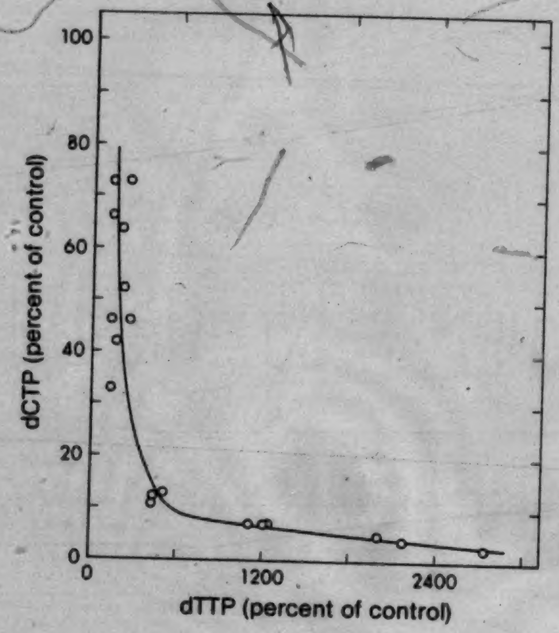

Figure 1. Relationship between concentrations of dTTP and those of dCTP. Control values (pmole/10^6 cells): dTTP, 58; dCTP, 223.

mM thymidine are shown in Fig. 2, and it is seen that a very rapid drop in dCTP occurred as dTTP increased. A similar experiment using 100 μM thymidine (not shown) produced similar deoxyribonucleoside triphosphate pool size changes, in that dCTP declined to 13% of control values and dTTP rose to 500% of control values within 2 h. In a separate experiment, 4.0 mM thymidine caused the dCTP pool to fall to 15% of the control by 0.5 h and to 6% of the control by 1 h.

Effect of dTTP on GDP Reduction

The model of ribonucleotide reduction predicts that an increase in dTTP concentrations will stimulate the rate of reduction of GDP, and that a decrease in dTTP will effectively inhibit its reduction. Changes in the rate of reduction of GDP should therefore be reflected in changes in the concentration of dGTP.

The data in Fig. 3 indicate a direct correlation between the concentrations of dTTP and those of dGTP. Under these conditions, the GTP concentration did not change. The lowest values presented for dTTP were 70 to 80% of control values, which corresponded to dGTP values of 50 to 55% of control. Lower dTTP concentrations would be necessary to properly determine the effect of a decrease in dTTP on dGTP.

Time course experiments using 2 mM thymidine also demonstrated that an increase in dGTP levels coincided with an increase in dTTP (Fig. 4). 100 μM thymidine pro-

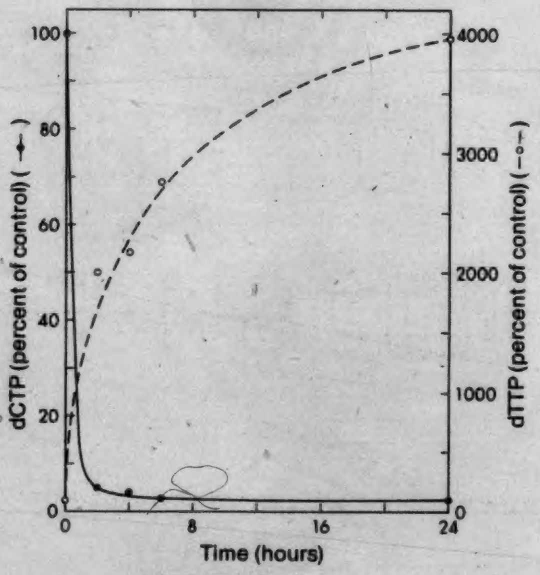

Figure 2. Effect of incubation with 2 mM thymidine on concentrations of dTTP (○) and dCTP (●).

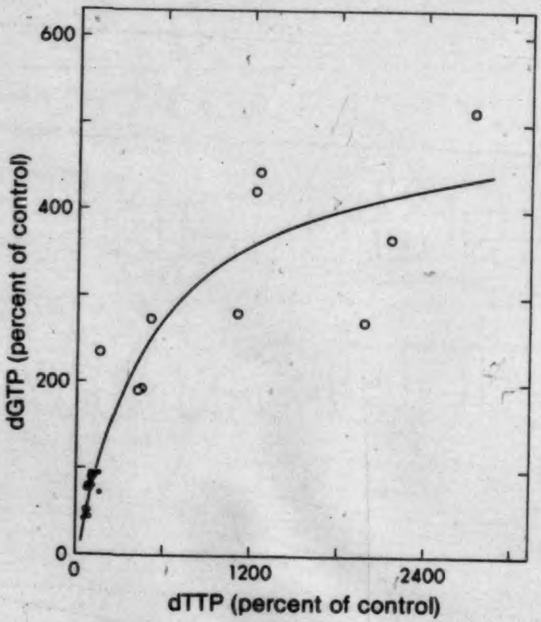

Figure 3. Relationship between concentrations of dTTP and those of dGTP. Control values (pmole/10^6 cells): dTTP, 58; dGTP, 12.

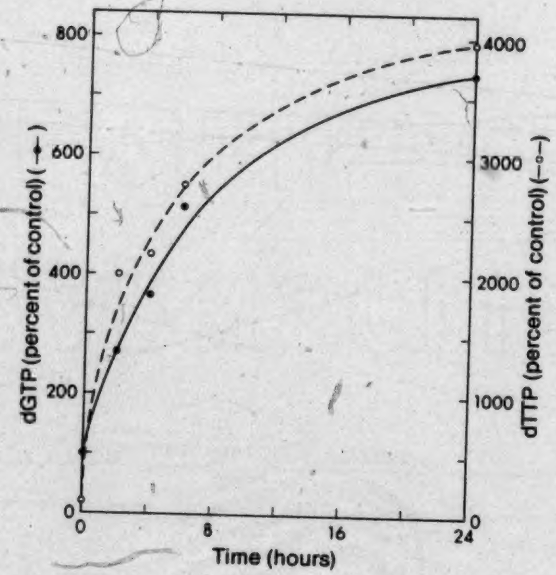

Figure 4. Effect of incubation with 2 mM thymidine on concentrations of dTTP (○) and dGTP (●).

duced a similar pattern of results, although the increases in dTTP and dGTP were 500% and 270% of control values, respectively.

Effect of dGTP on CDP Reduction

The model of ribonucleotide reductase regulation predicts that an increase in the concentration of dGTP will inhibit the reduction of CDP, while a decrease will stimulate CDP reduction. These changes should be reflected in the intracellular concentration of dCTP.

The data in Fig. 5 show that an increase in dGTP correlated with a decrease in dCTP. As well, a decrease in dGTP to 50% of control values corresponded to a substantial increase of dCTP to 200% of control values. These conditions did not change CTP concentrations.

The data in Fig. 6 show the temporal relationship between the increase of dGTP and the decrease in dCTP in the presence of 2.0 mM thymidine. 100 μM thymidine produced similar results (not shown), with dGTP levels at 270% of control and dCTP levels at 13% of control after 2 h.

Effect of dGTP on UDP Reduction

The ribonucleotide reductase model predicts that a decrease in dGTP concentrations should stimulate the reduction of UDP, thus causing an increase in dTTP concentrations.

The data in Fig. 7 indicate that a decrease in the

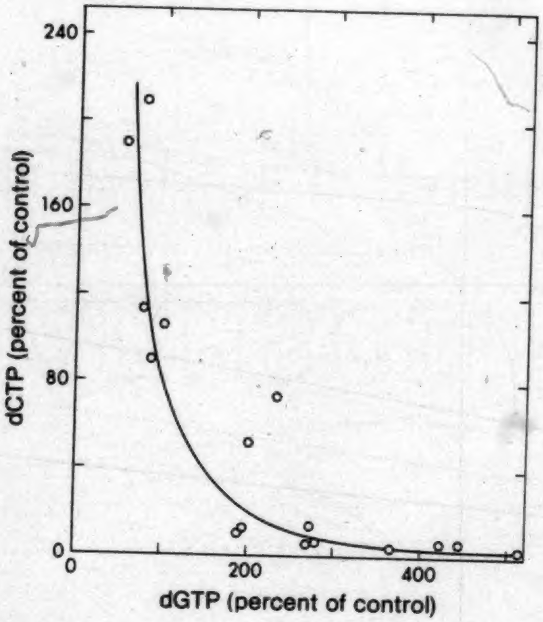

Figure 5. Relationship between concentrations of dGTP and those of dCTP. Control values (pmole/10^6 cells): dGTP, 12; dCTP, 223.

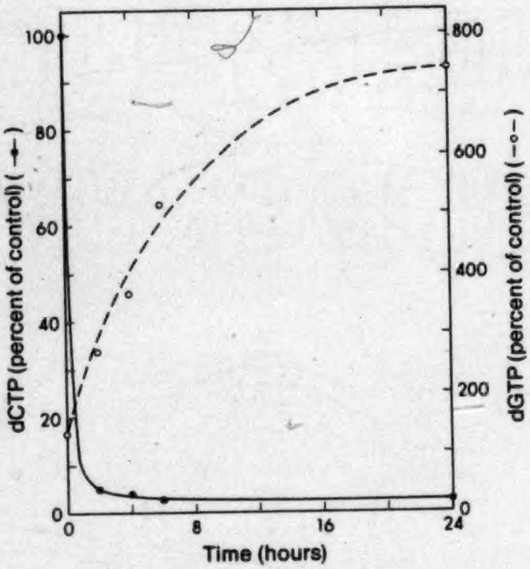

Figure 6. Effect of incubation with 2 mM thymidine on concentrations of dGTP (○) and dCTP (●).

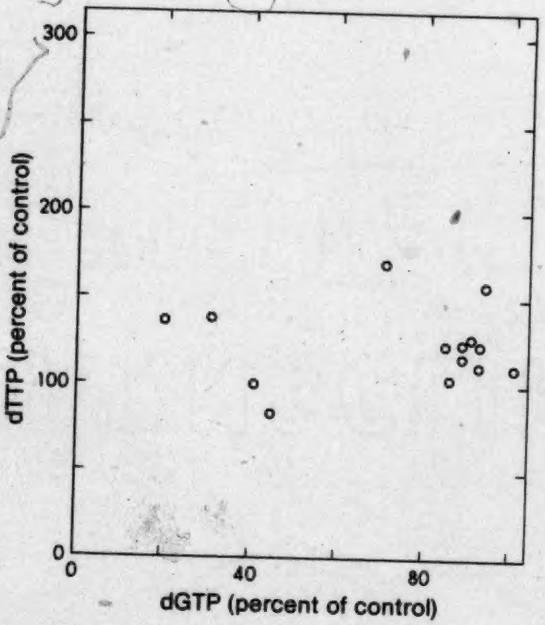

Figure 7. Relationship between concentrations of dGTP and those of dTTP. Control values (pmole/10^6 cells): dGTP, 12; dTTP, 58.

dGTP concentration did not correlate with a change in the dTTP pool. The conditions used had no effect on the UTP pool.

Effect of dGTP on ADP Reduction

The model being evaluated predicts that an increase in dGTP concentrations will increase the rate of reduction of ADP, while lowered dGTP concentrations will decrease the reduction of ADP. The changes should be reflected in the concentrations of dATP.

The data in Fig. 8 are more easily analyzed if they are identified as to the drug used to obtain them. When thymidine was used to manipulate the dGTP concentration, there was no increase in dATP with increasing dGTP concentration. When 3-deazauridine was used, dGTP concentrations of slightly less than control were obtained, but the points fall on a line parallel to the Y-axis, which actually indicates that a change in dATP had no effect on the dGTP concentration. Finally, when mycophenolic acid was used to lower dGTP concentrations, the points also fall on a line parallel to the Y-axis and again indicate that a change in dATP did not correlate with a change in dGTP. None of these manipulations caused a change in ATP concentrations.

Effect of dATP on CDP Reduction.

The final question posed in this study was: What is the effect of a change in dATP concentrations on dCTP concentrations? The ribonucleotide reductase model is not

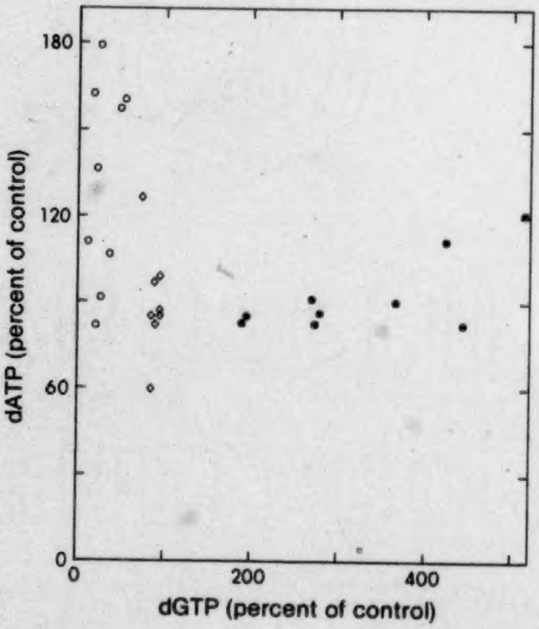

Figure 8. Relationship between concentrations of dGTP and those of dATP. dGTP concentrations were manipulated using 3-deazauridine (◇), thymidine (●), or mycophenolic acid (○). Control values (pmole/10^6 cells): dGTP, 12; dATP, 39.

easy to interpret on this point since it appears that an increase in dATP, with no change in ATP, could cause an increase, a decrease, or no change in the dCTP levels. This ambiguity arises because the model predicts that an increase in dATP levels increases the reduction of pyrimidines relative to purines, while it also decreases the overall activity of the enzyme.

The data presented in Fig. 9 are too scattered to allow a conclusion to be drawn as to the correlation or lack thereof between changes in dATP and changes in dCTP. Identification of the conditions used to manipulate dATP concentrations did not clarify the situation.

The conditions used had no effect on CTP concentrations.

DISCUSSION

This attempt to evaluate the relevance of the Thelander-Reichard model (and of other models) of ribonucleotide reductase regulation by deoxyribonucleoside triphosphates, for the reduction process in intact CHO cells, is based on the assumption that changes in deoxyribonucleoside triphosphate concentrations reflect changes in the rate of ribonucleotide reduction. Though reasonable in general terms, it must be pointed out that this assumption really has not been unequivocally proven.

The results shown in Fig. 1 and 2 are consistent with the model, which states that an increase in dTTP will in-

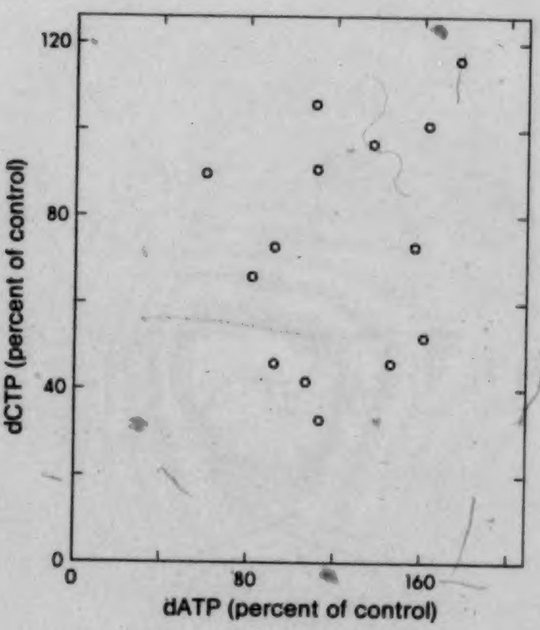

Figure 9. Relationship between concentrations of dATP and those of dCTP. Control values (pmole/10^6 cells): dATP, 39; dCTP, 223.

hibit CDP reduction. However, under these conditions, both the dTTP and the dGTP pools are elevated; therefore it is possible that dTTP or dGTP or both inhibit CDP reduction.

Even if elevated levels of dTTP result in a decrease in dCTP concentrations, possible causes other than inhibition of ribonucleotide reductase must be considered. Elevated dTTP might stimulate catabolism of dCTP or conversion of dCMP to dUMP. The latter possibility is unlikely since purified dCMP deaminase is inhibited by dTTP (16).

It is interesting that it was not possible to reduce the dCTP pool to less than 3% of control values, which in absolute values is approximately the same size as the control dGTP pool. The most likely explanation is that when dCTP concentrations decline to a certain level, inhibition of DNA synthesis results and a new steady-state dCTP concentration is established.

Lowe and Grindey (12) using L1210 cells, Grindey _et al_. (13) using CCRF-CEM cells, and Tattersall _et al_. (14) using PHA-stimulated human lymphocytes, all found that elevation of dTTP was correlated with a decrease in dCTP.

The results shown in Fig. 3 and 4 are consistent with the model which states that an increase in dTTP will stimulate reduction of GDP, while a decrease in dTTP will inhibit GDP reduction.

The plot of dTTP versus dGTP (Fig. 3) is steep at dTTP concentrations less than 600% of control and then begins to level off. There are three plausible explanations for the leveling-off of the curve:

1) dGTP may inhibit GDP reduction. This is consistent with the model under consideration and also agrees with the model proposed by Moore and Hurlbert (1) and Eriksson *et al*. (6), but is not consistent either with the model proposed by Larsson and Reichard (2,3) in which dGTP stimulates GDP reduction, or with the model proposed by Reichard (5) in which dGTP has no effect on GDP reduction. If dGTP does inhibit GDP reduction, one could not describe this as a tightly regulated system since dGTP reaches concentrations 400 to 500% of control.

2) The maximum rate of GDP reduction may have been reached.

3) dGTP catabolism may increase at high dGTP concentrations.

Others have also found that an increase in dTTP concentration correspond to an increase in dGTP concentration (12, 13,14).

The results shown in Fig. 5 and 6 are consistent with the model which states that an increase in dGTP will inhibit CDP reduction while a decrease in dGTP will stimulate CDP reduction. As discussed above, it was not possible to distinguish between the effects of elevated dTTP and elevated dGTP on the dCTP pool.

The results shown in Fig. 7 are not consistent with the model, which states that a decrease in dGTP will stimulate UDP reduction. This does not necessarily mean that the model is incorrect because there are more factors affecting the dTTP pool size than there are affecting the other deoxyribonucleoside triphosphate pools. Deoxyuridylate, the precursor of deoxythymidylate, is synthesized both from UDP and from dCMP. Allosteric regulation of thymidylate synthetase has not been reported, but purified dCMP deaminase is inhibited by dTTP and stimulated by dCTP (16). Therefore, it is possible that dGTP does inhibit UDP reduction *in vivo*, but that increased deamination of dCMP counteracts this effect. In a study of the reversal of deoxyguanosine toxicity by deoxycytidine in L5178Y cells, Theiss *et al.* concluded that dGTP inhibited UDP and CDP reduction and that addition of deoxycytidine supplied the dTTP pool via dCMP deaminase and thymidylate synthetase (17). However, this study was complicated by the fact that pool size measurements were not made.

The results shown in Fig. 8 also are not consistent with the model, which states that an increase in dGTP will stimulate ADP reduction whereas a decrease in dGTP will inhibit it. The possibility was considered that changes in dTTP might cause changes in dATP because the ribonucleotide reductase models of Moore & Hurlbert (1) and Larsson & Reichard (2,3) included a stimulation of ADP reduction by dTTP. However, no correlation was found (not shown). We have concluded that in CHO cells the concentration of dATP is not controlled by that of dGTP.

Grindey *et al*. (13) observed that in CCRF-CEM cells, elevated dGTP coincided with elevated dATP, whereas Tattersall (14) using PHA-stimulated human lymphocytes, found that a slightly elevated dGTP concentration corresponded to very low dATP concentrations.

The data obtained with 3-deazauridine and mycophenolic acid, which are also presented in Fig. 8, indicate that a change in dATP does not cause a change in dGTP. This is contrary to the ribonucleotide reductase hypothesis which states that an increase in dATP will inhibit GDP reduction. These results differ from those of Tattersall *et al*. (14) who found that elevated dATP corresponded to lowered dGTP, but agree with those of Lowe *et al*. (12) who found that elevated dATP corresponded to control concentration of dGTP.

Finally, the results shown in Fig. 9 do not allow the model to be tested as to whether or not dATP affects CDP reduction. The extent of scatter of the data probably indicates that there are other variables on which the dCTP and/or dATP concentrations depend. Lowe *et al*. (12) and Tattersall *et al*. (14) found that elevated dATP corresponded to lowered dCTP.

In this study we have attempted to dissect one of the recent models for the regulation of ribonucleotide reductase, namely that of Thelander and Reichard (7), and to test each part of the model separately, in cultured cells. There were several problems with this approach, including the difficulty in choosing conditions which allow relatively specific manipulations of a given pool. Another problem concerns ribonucleotide reductase itself, in that if part or all of this model is correct, a change in the concentration of one effector may cause a chain reaction of events, the final outcome of which may not be intuitively obvious by considering the model. For example, if the model were correct, an increase in dTTP concentration would cause a decrease in dCTP and an increase in dGTP, and the increase in dGTP would cause an increase in dATP. If the increase in dATP were relatively small, the enzyme would become pyrimidine specific and thus stimulate CDP and UDP reduction; however, if the increase in dATP were larger, all reductions would be inhibited. We have not observed this chain reaction in CHO cells since dGTP does not stimulate ADP reduction.

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CHAPTER 10

RELATIONSHIP BETWEEN RIBO- AND DEOXYRIBONUCLEOTIDE CONCENTRATIONS AND BIOLOGICAL PARAMETERS IN CULTURED CHINESE HAMSTER OVARY CELLS

INTRODUCTION

Inhibition of cell growth and loss of cell viability are associated with altered intracellular nucleotide concentrations following treatment with a number of purine and pyrimidine antimetabolites, some naturally occurring purines and pyrimidines, and other agents that affect purine and pyrimidine metabolism. This qualitative relationship between nucleotide concentrations and important biological parameters has been extended in some cases to a quantitative or semi-quantitative relationship between changes in concentrations of one or another nucleotide and inhibition of growth (e.g., ref. 1-4); however, only in recent years have concentrations of all the purine and pyrimidine ribo- and deoxyribonucleotides been regularly measured in such studies. In contrast to studies that have measured growth inhibition, few if any detailed investigations have been made of the relationship(s) between nucleotide concentrations and loss of cell viability.

In an attempt to study the quantitative relationships among nucleotide concentrations, cell growth and cell viability, we have studied growth rate, cloning efficiency, nigrosin exclusion and progression through the cell cycle, in cultured Chinese hamster ovary cells in which nucleotide concentrations were altered in several different ways.

MATERIALS AND METHODS

Materials. Mycophenolic acid was a gift of Dr. T.J. Franklin. PALA (phosphonoacetyl-L-aspartate) and pyrazofurin were obtained from the Division of Cancer Treatment, U.S. National Cancer Institute. Purine and pyrimidine bases, nucleosides and nucleotides were obtained from the Sigma Chemical Co. [8-^{3}H]dATP (10 Ci/mmole), [8-^{3}H]dGTP (19 Ci/mmole), [5-^{3}H]dCTP (20 Ci/mmole) and [methyl-^{3}H]dTTP (17 Ci/mmole) were purchased from Schwarz-Mann. Poly [d(AT)] and poly [d(IC)] were purchased from the Sigma Chemical Co. and from Miles Laboratories, respectively. *E. coli* DNA polymerase I (E.C.2.7.7.7; 2500-5000 units/mg) was purchased from Boehringer Mannheim Corp.

Cell Culture. Chinese hamster ovary-K1 cells, obtained from Dr. G. Whitmore (Ontario Cancer Institute, Toronto, Canada), were grown in alpha-MEM medium containing 10% dialyzed fetal calf serum (Grand Island Biological Co.). The cells were grown in 125 ml bottles on a Model G-2 Gyrotatory shaker (New Brunswick Scientific Co.) at 200 rpm. The average doubling time was 12 hr and the growth rate was exponential to 0.8-1.0 x 10^{7} cells/ml. The cells were routinely tested for mycoplasma and found to be negative.

Biological Parameters. Cell density and the population volume distribution were determined using a Model Z_F Coulter Counter equipped with a 100-channel Coulter Channelyzer II. The cell cycle distribution was determined by flow microfluorometry using a Bio/Physics Model 4800A

Cytofluorograph equipped with a 100-channel Model 2100 pulse height analyzer. Cells were collected in a clinical centrifuge, resuspended to 400,000 cells/ml in 0.05 mg/ml propidium iodide in 0.1% sodium citrate, and stained for 20 to 40 min on ice. In any given experiment, the staining time was identical for all the samples.

Cell viability was determined using a simple colony forming assay as follows: cell density was measured, followed by three serial dilutions using warm, gassed media, to give a final cell density of 50-100 cells/ml for untreated cells. When necessary, higher final densities were used for drug-treated cells to allow accurate determination of cell kill. 2 ml of cell suspension were added to each of 3 warmed, gassed 25 cm^2 plastic tissue culture flasks. After incubation for 7 days at 37°C in a humidified atmosphere of 5% CO_2 in air, the medium was poured off, the colonies stained with 0.1% crystal violet in physiological saline for ca. 30 min, and then counted. Control cloning efficiencies were routinely greater than 90%.

Cell integrity at the time of cloning was determined for each drug treatment condition by measuring nigrosin exclusion.

Nucleotide Concentrations. Methods for the extraction of nucleotides from cultured cells, and for the measurement of ribonucleotide and deoxyribonucleotide concentrations have been described previously.

RESULTS

In order to study relationships between changes in nucleotide concentrations and biological parameters, it was necessary first to establish conditions under which both nucleotide concentrations and growth inhibition varied over a wide range. Nucleotide pools were therefore manipulated using mycophenolic acid, an inhibitor of inosinate dehydrogenase (E.C.1.2.14) (5,6); pyrazofurin, an inhibitor of orotidylate decarboxylase (E.C.4.1.1.23) (7); PALA, an inhibitor of aspartate transcarbamylase (E.C.2.1.3.2) (8); and thymidine.

Table 1 shows the amount of growth inhibition and loss of cell viability caused by these agents at several concentrations of these agents. At the time of cloning nigrosin exclusion was also measured and it was found that more than 90% of the cells excluded dye under all conditions; this demonstrates that the cells being cloned were intact. As well, the debris peak on the Coulter Channelyzer was approximately the same size in both treated and control cells indicating that cell lysis was not occurring. Finally, the ratio of ATP to ADP was determined by HPLC on cell extracts prepared at the time of cloning; it was approximately the same for both control and treated cells (>10:1), thus indicating that the energy status of the cells was normal. The treatment conditions chosen for more detailed study were: 2 μM mycophenolic acid, 0.2 μM pyrazofurin,

Table 1

Effect of several drug treatment conditions on growth rate and viability of CHO cells

Treatment condition	Growth rate	Cloning efficiency
	(Percent of control)	
0.1 μM Mycophenolic acid; 25 hr	100	100
0.5 μM Mycophenolic acid; 25 hr	67	78
1.0 μM Mycophenolic acid; 24 hr	21	32
2.0 μM Mycophenolic acid; 22 hr	17	4
10.0 μM Mycophenolic acid; 25 hr	0	0
0.01 μM Pyrazofurin; 24 hr	83	95
0.04 μM Pyrazofurin; 20 hr	71	N.D.
0.1 μM Pyrazofurin; 20 hr	56	N.D.
0.2 μM Pyrazofurin; 22 hr	30	49
2.0 μM Pyrazofurin; 24 hr	35	25
100 μM PALA; 24 hr	84	89
500 μM PALA; 22 hr	40	41
100 μM Thymidine; 24 hr	100	N.D.
500 μM Thymidine; 24 hr	79	N.D.
2.0 mM Thymidine; 24 hr	63	86

(cont'd from Table 1 ...)

For all treatment conditions more than 90% of the cells excluded dye at the time of cloning.

Growth rates are expressed as the average growth rate from the time of addition of drug.

The values for untreated cells for growth rate and cloning efficiency were 1 doubling per 12 hr and 90%, respectively.

500 μM PALA, and 2.0 mM thymidine.

The next step was to relate nucleotide pool sizes to growth rate and cell viability. Table 2 compares these parameters determined during treatment of CHO cells with 2.0 μM mycophenolic acid. Changes occurred in all the nucleoside triphosphate pools, with the largest changes in the GTP, dGTP, dTTP and dATP pools. The GTP and dGTP pool sizes changed very little after the first 2 hr of treatment, whereas the dTTP pool continued to increase after 6 hr of treatment. A substantial change also occurred in the dATP pool between 6 and 22 hr of treatment with a decrease from 163% to 57% of control. The cell viability was not affected after 2 hr of treatment, only decreasing to 86% of control after 4 hr.

The only pool size change which correlated well with the decrease in viability was the increase in dTTP. The linear correlation coefficient for this relationship was 0.996. In order to determine if the loss of viability could be related to an increase in dTTP, the dTTP pool was elevated to 400-500% of control using 100 μM thymidine; however this condition was not growth inhibitory; therefore the loss of cell viability caused by mycophenolic acid cannot be explained simply on the basis of an increase in the dTTP pool.

In order to determine the effect of the nucleotide pool size changes caused by mycophenolic acid on the progression of cells through the cell cycle, the cell cycle

Table 2

Nucleotide pools, growth rate, and viability during treatment of CHO cells with 2.0 μM mycophenolic acid

Treatment Time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency
	(Percent of control)									
2	96	30	94	119	82	21	66	137	N.D.	106
4	81	17	123	142	111	15	91	179	N.D.	86
6	88	18	118	146	163	18	101	214	58	66
22	124	29	135	139	57	31	126	300	0	4

Nucleotide values for untreated cells, in pmoles per 10^6 cells, were: ATP, 5580; GTP, 1070; CTP, 1230; UTP, 2390; dATP, 39; dGTP, 12; dCTP, 223; and dTTP, 58.

distribution of the population was measured. After 2 hr of treatment, there was a very small increase in the number of cells in G_1 and a small decrease in the number of cells in G_2. By 6 hr of treatment, more than 70% of the cells had accumulated near the G_1/S border, and by 22 hr, this increased to 80% with most of the remaining cells in S phase.

No change in modal cell volume of the population had occurred by 6 hr of treatment; however, by 22 hr the volume had increased to 167% of control.

Table 3 compares nucleotide pool sizes with cell growth rate and cell viability during treatment with 0.2 μM pyrazofurin. Changes occurred in all the nucleoside triphosphate pools with the largest changes occurring in the CTP, UTP and dCTP pools. The changes in these pools were small after the first 2 hours of treatment. As observed with mycophenolic acid treatment, the dATP pool decreased between 6 and 22 hr of treatment. The growth rate was unaffected at 6 hr of treatment, but the cloning efficiency had decreased to 78% of control. Between 6 and 22 hr both the growth rate and viability decreased substantially, and the largest nucleotide pool size change was the decrease in dATP. None of the changes in the nucleotide pools correlated well with the loss of viability over the period studied.

Flow microfluorometry showed only small changes in the cell cycle distribution during the first 6 hr of treat-

Table 3

Nucleotide pools, growth rate, and viabilitty during treatment of CHO cells with 0.2 μM pyrazofurin

Treatment Time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency
	(Percent of control)									
2	119	128	16	25	112	50	31	81	N.D.	91
4	111	109	2	7	109	42	13	74	N.D.	78
6	108	106	3	7	113	43	13	83	106	78
22	134	127	9	10	53	46	11	68	17	41

ment, with a small decrease (ca. 6%) in the proportion of cells in late S and G_2 and a corresponding increase in cells in G_1 or early S phase. Since there was no growth inhibition during the initial 6 hr of treatment, the cells must have been dividing at control rates but not progressing as fast through S phase. There was little or no change in the distribution between 6 and 22 hr. Therefore, unlike mycophenolic acid, pyrazofurin did not cause substantial synchronization of the cells, probably because cell growth was not completely inhibited.

No changes in modal cell volume had occurred after 6 hr of treatment and by 22 hr the modal volume had only increased to 125% of control.

Table 4 compares nucleotide pool sizes with growth rate and cell viability during treatment with 500 μM PALA. As with pyrazofurin treatment, the largest changes were in the CTP, UTP and dCTP pools, but substantial decreases also occurred in the dGTP and dTTP pools; these decreased to 21% and 41% of control, respectively. Again as with pyrazofurin treatment, the growth rate was unaffected after 6 hr of treatment, but the viability had decreased to 67% of control. Between 6 and 22 hr, both the growth rate and viability declined to approximately 40% of control.

Both pyrazofurin and PALA inhibit pyrimidine synthesis de novo and, although the results from the two drugs were qualitatively similar, there were quantitative differences between them; thus under pyrazofurin treatment, a growth

Table 4

Nucleotide pools, growth rate, and viability during treatment of CHO cells with 500 μM PALA

Treatment Time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning efficiency
	(Percent of control)									
2	96	103	64	62	96	79	59	79	N.D.	109
4	112	110	43	34	103	52	40	81	N.D.	82
6	110	107	24	19	94	51	25	77	101	67
22	88	132	9	9	78	21	13	41	40	41

rate of 17% of control corresponded to a cloning efficiency of 41% of control, whereas under PALA treatment, a growth rate of 40% of control corresponded to 40% cloning efficiency. In addition, the minimum value for the dGTP pool size was 42% of control with pyrazofurin treatment, twice the minimum value achieved with PALA treatment. Pyrazofurin was much more potent than PALA in CHO cells; 0.2 μM pyrazofurin reduced the UTP pool to 25% and 10% at 2 hr and 22 hr of treatment, respectively, while 500 μM PALA reduced the UTP pool to 62% and 9% of control at 2 hr and 22 hr, respectively.

Unlike pyrazofurin, PALA caused no change in cell cycle distribution at 6 hr of treatment, probably because of the slower rate of decrease in the concentrations of CTP, UTP and dCTP in PALA treated cells. By 22 hr, there was a small decrease (ca. 6%) in the proportion of cells in the late S and G_2 phases with a corresponding increase in G_1 or early S.

There was little (40%) increase in modal cell volume even after 22 hr of treatment.

In order to determine the effects of varying only deoxyribonucleotide concentrations on cell growth rate and viability, cells were treated with thymidine (Table 5). The main effects observed were very large increases in dTTP and dGTP, and a large decrease in dCTP concentrations. Although 2 hr treatment produced lower dCTP levels than were achieved at any time with pyrazofurin or PALA, the minimum growth

Table 5

Nucleotide pools, growth rate, and viability during treatment of CHO cells with 2.0 mM thymidine

Treatment Time (hr)	ATP	GTP	CTP	UTP	dATP	dGTP	dCTP	dTTP	Growth rate	Cloning* efficiency
	(Percent of control)									
2	84	82	84	84	92	269	5	2000	N.D.	N.D.
4	69	77	73	72	91	367	4	2164	N.D.	N.D.
6	91	97	88	95	122	514	3	2745	63	101
24	145	141	111	102	146	741	3	3692	63	86

*Cloning efficiency and nucleotide pool sizes were determined in separate experiments. The growth rates in the experiments in which nucleotide pools and cloning efficiency were measured were 48 and 63%, respectively.

rate achieved was still 63% of control, and the viability was still 86% of control after 22 hr of treatment. As well, it should be noted that 100 μM thymidine (which is not growth inhibitory), resulted in dTTP and dGTP concentrations of 500% and 200% of control, respectively, and dCTP concentrations of 12% of control; this was the lowest level of dCTP achieved by pyrazofurin and PALA treatment.

2.0 mM thymidine inhibited the progression of the cells through the cell cycle with most of the cells in G_1 or early S phase; however, between 6 and 22 hr the cells obviously overcame the block because by 22 hr the cell cycle distribution was similar to control with only a slightly smaller proportion of cells in the G_1 and G_2 phases.

After 6 hr of treatment, there was no change in the modal cell volume of the population, although substantial synchronization had occurred; however by 22 hr the modal cell volume was approximately 180% of control.

DISCUSSION

The relationship between nucleotide concentrations and biological parameters obviously is complex, and appears to vary both with respect to the nucleotide changes involved and with respect to the biological parameter studied.

However, inhibition of cell growth can be correlated, at least in general terms, with decreases in the concentration of one or another ribo- or deoxyribonucleoside triphosphate. Thus treatment with a growth inhibitory concentration of mycophenolic acid resulted in substantial decreases in both the GTP and dGTP pools, which logically may be expected to have consequences for both RNA and DNA synthesis. We have found that after 2 hr of treatment with 2 µM mycophenolic acid, DNA and RNA synthesis were inhibited by 80 and 73%, respectively (D. Hunting and J.F. Henderson, unpublished).

The growth inhibition caused by pyrazofurin and PALA can also be rationalized in terms of an inhibition of DNA and RNA synthesis, since both these drugs caused large decreases in the concentrations of CTP, UTP and dCTP. We have found that after 2 hr of treatment with 0.2 µM pyrazofurin, DNA and RNA synthesis were inhibited by 60 and 46%, respectively (D. Hunting and J.F. Henderson, unpublished). Finally, the slight inhibition of growth caused by 2.0 µM thymidine could be a result of an inhibition of DNA synthesis caused by the large decrease in the concentration of dCTP (3,9-11), although it is equally plausible that the elevated dTTP and dGTP inhibit DNA synthesis. The fact that 100 µM thymidine caused large changes in the concentrations of dTTP, dGTP and dCTP without inhibiting growth may indicate that these cells are relatively insensitive to changes in deoxyribonucleotide concentrations in the absence of changes in ribonucleotide concentrations.

The effects of the various treatment conditions on progression through the cell cycle are not as readily explained in terms of changes in nucleotide concentrations.

Thus mycophenolic acid caused an accumulation of cells in late G_1 or early S phase, while pyrazofurin caused little synchronization under strongly growth inhibitory conditions. PALA also caused little synchronization under growth inhibitory conditions. These results suggest that a decrease in the concentrations of GTP and dGTP may inhibit a specific step in the progression of cells through the cell cycle, while a decrease in the concentration of pyrimidine ribo- and deoxyribonucleoside triphosphates causes a relatively non-specific inhibition of progression through the cell cycle.

Thymidine, however, caused substantial synchronization at early treatment times, but much less synchronization at later times. This change in the degree of synchronization corresponded to relatively minor changes in nucleotide concentrations. It seems likely that time was an important variable, but further study will be necessary to determine which time-dependent process allowed the cells to overcome the specific block in progression through the cell cycle.

The relationship between nucleotide concentrations and loss of cell viability is complex. First, in no case studied were altered nucleotide concentrations associated with decreased dye exclusion. This finding probably is related to the observation that under none of the conditions used was the ATP:ADP ratio lowered appreciably. A reasonable interpretation of these results is that at the time of cloning all the cells were metabolically healthy, but that the cells with low cloning efficiency had suffered permanent damage to their reproductive capacity during drug treatment.

Although in the case of mycophenolic acid treatment, the loss of viability correlated with increases in dTTP concentrations, such a simple cause and effect relationship is unlikely; thus much larger increases in dTTP produced during thymidine treatment, had little effect on cloning efficiency. No other correlations of nucleotide concentration changes with cloning efficiency were observed, and it seems likely that cell death is related not only to changes in the concentration of one or another nucleotide, but also to the length of time that the nucleotide pool imbalances are abnormal.

Lowe et al. concluded that mycophenolic acid induced "unbalanced growth" in L5178Y cells because protein and RNA synthesis were inhibited less than DNA synthesis (1). This explanation does not apply to cell death in CHO cells as induced by mycophenolic acid since RNA and DNA are inhibited by similar amounts. As well, the phenomenon of "unbalanced growth" resulting in cell death is in itself not understood.

Clearly, further studies of the relationship between nucleotide pool size changes and cell death are required.

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